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**THERAPEUTIC ANTIBODY AGAINST *NEISSERIA*
GONORRHOEAE LIPOOLIGOSACCHARIDE, A PHASE-
VARIABLE VIRULENCE FACTOR**

A Dissertation Presented

By

SRINJOY CHAKRABORTI

Submitted to the Faculty of the University of Massachusetts
Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

MAY 25TH 2017

BIOMEDICAL SCIENCES

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ACKNOWLEDGEMENTS

Graduate school has been arduous and exhilarating. The constants through this tumultuous odyssey have been the unwavering encouragement and love of my parents, Suktisuvra and Sibsankar Chakraborti. I would also like to thank my extended family for their continued support. In particular, I would like to acknowledge two of my cousins Drs. Abhiroop and Anandaroop Mukhopadhyay for motivating me to get a Ph.D. and for guiding me through the process of graduate school applications.

My thesis advisor Dr. Sanjay Ram has shown an immense amount of patience and confidence in me, and for that, I am grateful. I thank him for his continued encouragement, support, and guidance. He has taught me laboratory techniques, instilled in me the ability to analyze and criticize my own data, and helped me hone my communication skills. I am especially thankful that he has always willingly engaged in scientific thought exercises which have over time enabled me to distinguish between testable hypotheses and outlandish ideas.

I thank Drs. Peter Rice, Egil Lien, Christopher Sassetti, Jon Goguen, and Brian Akerley for their invaluable advice, guidance, and their time throughout my graduate career especially at my Thesis Research Advisory Committee (TRAC) meetings. I would also like to thank Drs. Ashild Vik, Alison Criss, Daniel Stein, and colleagues at Genmab B.V. (The Netherlands) for providing reagents and protocols.

My friends, in particular, Pia Negroni, Ken-Edwin Aryee, Juahdi Monbo, Priyanka Sinha, and Sunil Malonia have made graduate school and these past few years in Worcester memorable. My high school buddies, Sumit Dey and Dr.

Angshuman Maulik have been unconditionally supportive and I have treasured their counsel. Dr. Marilena Ioana Marinescu is a great friend, and her support and companionship have been a source of strength and stability through tough times in life and graduate school.

I acknowledge Nancy Nowak, Sunita Gulati, Bo Zheng, Rosane de Oliveira, Samuel Fountain, Jutamas Shaughnessy, Lisa Lewis, and colleagues at National Research Council-Canada for their help. I thank Drs. Sarika Agarwal and Shreekant Vasudhev for onboarding me into the laboratory. I am also grateful to colleagues in the Department of Medicine for teaching me techniques, providing me reagents, and for being supportive of my endeavors. I have thoroughly enjoyed working alongside them. I am especially thankful to Drs. Shruti Sharma, Samyabrata Bhaduri, and Maninjay Atianand for their guidance as I navigated my future direction after graduate school. I am also profoundly grateful to others I could not include in this non-exhaustive list.

A handwritten signature in black ink, appearing to read 'Srinjoy', with a stylized flourish extending from the end.

Srinjoy Chakraborti
Worcester, Massachusetts.
May 25th, 2017.

ABSTRACT

Neisseria gonorrhoeae (*Ng*) which causes gonorrhea has become multidrug-resistant, necessitating the development of novel therapeutics and vaccines. mAb 2C7 which targets an epitope within an important virulence factor, the lipooligosaccharide (LOS), is a candidate therapeutic mAb. Ninety-four percent of clinical isolates express the 2C7-epitope which is also a vaccine target.

Ng expresses multiple LOS(s) due to phase-variation (pv) of *LOS* glycosyltransferase (*lgt*) genes. mAb 2C7 reactivity requires a lactose extension from the LOS core Heptose (Hep) II (i.e. *lgtG* 'ON' [G+]). Pv results in HepI with: two (2-), three (3-), four (4-), or five (5-) hexoses (Hex). How HepI glycans impact *Ng* infectivity and mAb 2C7 function are unknown and form the bases of this dissertation.

Using isogenic mutants, I demonstrate that HepI LOS glycans modulate mAb 2C7 binding. mAb 2C7 causes complement (C')-dependent bacteriolysis of three (2-Hex/G+, 4-Hex/G+, and 5-Hex/G+) of the HepI mutants *in vitro*. The 3-Hex/G+ mutant (resistant to C'-dependent bacteriolysis) is killed by neutrophils in the presence of mAb and C'. In mice, 2- and 3-Hex/G+ infections are significantly shorter than 4- and 5-Hex/G+ infections. A chimeric mAb 2C7 that hyperactivates C', attenuates only 4- and 5-Hex/G+ infections.

This study enhances understanding of the role of Hepl LOS pv in gonococcal infections and shows that longer Hepl glycans are necessary for prolonged infections *in vivo*. This is the first study that predicts *in vitro* efficacy of mAb 2C7 against all four targetable Hepl glycans thereby strengthening the rationale for development of 2C7-epitope based vaccines and therapeutics.

LIST OF ABBREVIATIONS

°C	Degrees Centigrade
2C7-murine	Murine mAb 2C7
2C7-Ximab	Chimeric mAb 2C7
A	Alanine
Ab	Antibody
ADE	Antibody-dependent enhancement
Ag	Antigen
ANOVA	Analysis of variance
ASGP-R	Asialoglycoprotein receptor
ATCC	American Type Culture Collection
AUC	Area under the curve
bNAb	Broadly neutralizing Ab
C	Colistin
C'	Complement
C4BP	C4 binding protein
CA	Chocolate agar
CA-VCNTS	Chocolate agar with VCNTS
CD	Cluster of differentiation
CDC	Centers for Disease Control and Prevention
CEACAM	Carcinoembryonic antigen-related cell adhesion molecules
CFU	Colony forming units
C_H	Constant domain of Ig heavy chain
C_L	Constant domain of Ig light chain
CMP-Neu5Ac	Cytidine-5'-monophospho-N-acetylneuraminic acid
CO₂	Carbon dioxide
CR	Complement receptor
D	Aspartic acid

DENV	Dengue virus
DNA	Deoxyribonucleic acid
E	Glutamic acid
E430G Fc	2C7-Ximab E430G Fc
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
Erm	Erythromycin
FarAB	Fatty acid resistance system
FBS	Fetal bovine serum
Fc	Fragment, crystallizable (part of Ig)
FcγR	Fc γ Receptors (for IgG)
FCM	Flow cytometry
FDA	US Food and Drug Administration
FH	Factor H
FITC	Fluorescein isothiocyanate
Fv	Fragment, variable (part of Ig)
G	Glycine
Gal	Galactose
GalNAc	N-acetylgalactosamine
GGI	Gonococcal genetic island
Glc	Glucose
GlcNAc	N-acetylglucosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp	Glycoprotein
h	Hours
HBSS	Hank's balanced salt solution
Hep	Heptose
Hex	Hexose
HexNAc	N-acetylhexosamine

HIV	Human immunodeficiency virus
ID₅₀	Infectious dose ₅₀ (number of organisms required to infect 50% of the animals)
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl-β-D-thiogalactopyranoside
K	Lysine
Kan	Kanamycin
KDO	2-Keto-3-Deoxy-D- <i>manno</i> -octonic acid
KO	Knock-out
LbpA	Lactoferrin-binding protein A
LbpB	Lactoferrin-binding protein B
LDLRA	Low-density lipoprotein-receptor associated
Lf	Lactoferrin
<i>lgt</i> (gene)	<i>Lipooligosaccharide glycosyl transferase</i> gene
Lgt (protein)	Lipooligosaccharide glycosyl transferase enzyme
LHr	Lutropin receptor
LNnT	Lacto-N-neotetraose
LOS	Lipooligosaccharide
LPS	Lipopolysaccharide
M	Molar
mAb	Monoclonal antibody
MAC	Membrane attack complex
MACPF/CDC	MAC-perforin/cholesterol dependent cytolysin
MES	2-(<i>N</i> -morpholino) ethanesulfonic acid
min	Minutes
MisRS	Mg ²⁺ regulated <i>Neisserial</i> 2 component response system similar to PhoP/PhoQ

MLST	Multi-locus sequence typing
MOI	Multiplicity of infection
MS	Mass spectrometry
MsrAB	Methionine sulfoxide reductase A/B
<i>Mtb</i>	<i>Mycobacterium tuberculosis</i>
MtrCDE	Multiple transferable resistance system
Muc	Mucin
MutY	Mutator Y (causes G·C → T·A transversions in <i>E coli</i>)
MyD88	Myeloid differentiation primary response gene 88
N	Nystatin
N	Asparagine
NAATs	Nucleic Acid Amplification Tests
NET	Neutrophil extracellular traps
NF-κB	Nuclear Factor κ B
<i>Ng</i>	<i>Neisseria gonorrhoeae</i>
NG-MAST	<i>N. gonorrhoeae</i> multiantigen sequence typing
<i>Ng</i>PLD	Gonococcal phospholipase D
NHS	Normal human serum
NK	Natural Killer cells
NOD	Non-obese diabetic
Null Fc	2C7-Ximab Fc
OM	Outer membrane
Opa	Opacity proteins of <i>Ng</i>
OS	Oligosaccharide
P	Proline
PacA	Peptidoglycan O-acyltransferase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction

PEA	Phosphoethanolamine
PG	Peptidoglycan
PGCT	Peptidoglycan-derived cytotoxin
PI3K	Phosphoinositide 3-kinase
Pil	Pilin
PMNs	Polymorphonuclear leukocytes (or neutrophils)
Por	Porin
PRC	People's Republic of China
pv	Phase-variable
R	Arginine
RecA	Recombination protein A
Rmp	Reduction modifiable protein
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute medium
<i>scid</i>	Severe combined immunodeficiency
SDS	Sodium dodecyl sulfate
sec	Seconds
SEM	Standard error of mean
SIV	Simian immunodeficiency virus
Sm	Streptomycin
<i>sps.</i>	Species
SSM	Slipped strand mispairing
STD	Sexually transmitted diseases (now referred to as STI)
STI	Sexually transmitted infections
Tf	Transferrin
Tg	Transgenic
Th	T helper cells
TIFA	TNF- α receptor-associated factor-interacting protein with a forkhead-associated domain

TLR4	Toll-like Receptor 4
TNF-α	Tumor Necrosis Factor- α
TRIF	TIR-domain-containing adapter-inducing interferon- β
TS	Trimethoprim sulfate
TSP-1	Thrombospondin-1
U	Units
UK	The United Kingdom
US	United States of America
USD	US Dollar
Uvr	Ultraviolet resistance determinant
V	Vancomycin
WHO	World Health Organization
WT	Wild type
WT Fc	2C7-Ximab WT Fc
yrs.	Years

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LIST OF COPYRIGHTED MATERIALS

CHAPTER II of this thesis, entitled, “Phase-variable heptose I glycan extensions modulate efficacy of 2C7 vaccine antibody directed against *Neisseria gonorrhoeae* lipooligosaccharide”, was originally published in The Journal of Immunology. Chakraborti, S., L. A. Lewis, A. D. Cox, F. St Michael, J. Li, P. A. Rice, and S. Ram. 2016. Phase-Variable Heptose I Glycan Extensions Modulate Efficacy of 2C7 Vaccine Antibody Directed against *Neisseria gonorrhoeae* Lipooligosaccharide. *J Immunol* 196: 4576-4586. © [2016] The American Association of Immunologists, Inc.

CHAPTER I

Introduction

Etiology and epidemiology

Gonorrhea is the second most common bacterial sexually transmitted infection, and is caused by the pathogen *Neisseria gonorrhoeae* (*Ng* or gonococcus). While earliest references to the disease date back to the Old Testament, the second century physician Galen introduced the term gonorrhea to describe urethral exudates that he mistook for seminal discharge (*gonos* ['seed'] and *rhoia* ['flow']). Colloquially, gonorrhea is also called 'the clap', a phrase thought to have originated in 1378 from Les Clapiers, a district in Paris that was inhabited by sex workers (1-3).

Ng are Gram-negative (Fig. 1), facultatively anaerobic, non-motile (in the conventional sense; twitching motility mediated by pili is observed), non-sporulating cocci that grow in pairs (diplococci) (Fig. 1). Like other members of genus *Neisseria*, they are capnophilic (require 5% CO₂), and grow at temperatures between 35 – 37 °C. *Ng* are oxidase (tetra/di-methyl-p-paraphenylenediamine) positive, but differ from other members of the *Neisseria* spp. in their ability to reduce nitrates to nitrites, and in their ability to ferment only glucose.

An estimated 78.3 million new cases of gonorrhea occur worldwide each year (3). In 2012, the highest number of cases were reported from the WHO Western Pacific Region, followed by the African Region. The lowest incidence was observed in the Eastern Mediterranean and European Regions, while the South-

East Asia Region and the Region of the Americas reported intermediate incidence (3). Within the US, 395,216 cases were reported in the year 2015; a 12.8% increase compared to 2014, and a 19.9% rise over 2011 rates. Rates of gonorrhea increased by 18.3% and 6.8% in men and women, respectively. The highest rates of disease were recorded in the 20-24 yrs. age group for both sexes. Overall, the rate of infection in women was 107.2 per 100,000 people, whereas in men it was 140.9. Across all age groups, the highest rates of infection were observed amongst men who had sex with men, whereas women who had sex women accounted for the lowest rates. The highest number of cases occurred in the African American population, followed by American Indians/Alaska Natives and Native Hawaiians/Other Pacific Islanders. Less than hundred incidents per 100,000 people were recoded amongst Hispanics, Asians and Whites. Geographically, the Southern states reported the highest number of cases, followed by the West and the Midwest, whereas the Northeast had the least number of cases (4).

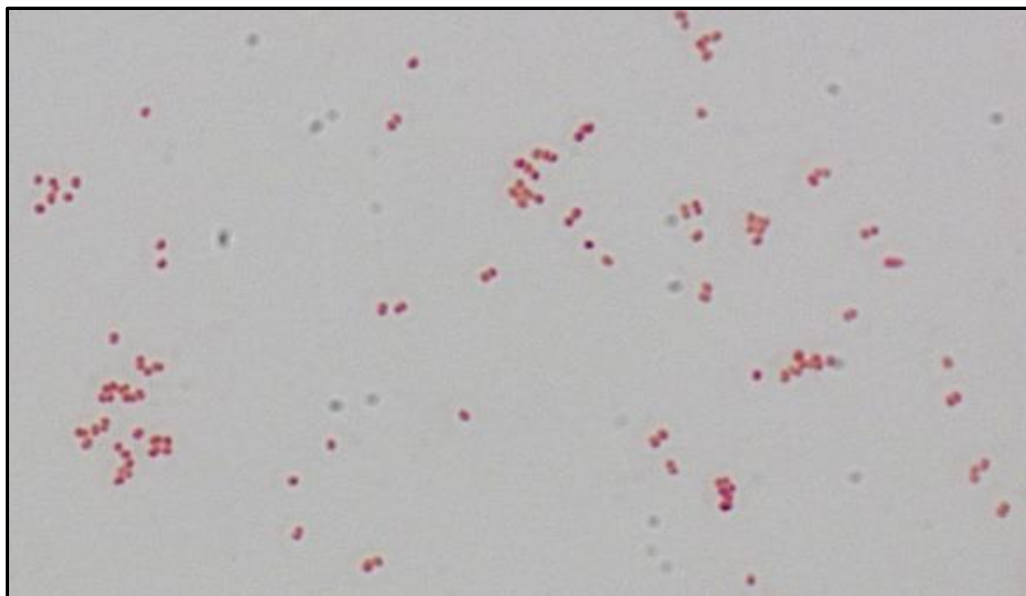


Figure 1. Gram-stained *Neisseria gonorrhoeae* (Ng). Ng are Gram-negative cocci that occur in pairs (diplococci). An isolated colony from an overnight culture of MS11 WT on chocolate agar was suspended in normal saline (0.9% NaCl) and spread on a clean glass slide. Subsequently, the smear was air dried and heat fixed. The slide was stained with crystal violet (1 min), fixed with Gram's iodine (1 min), decolorized with acetone-ethanol (1:1 ratio, 30 sec) and finally counterstained with safranin (45 sec). The smear was visualized under a 100x oil-immersion (1000x magnification).

Clinical symptoms

Uncomplicated gonorrhea is an acute, often self-resolving and localized infection, characterized by inflammation of the genitourinary mucosae including the urethra, vagina, endocervix, rectum, and pharynx (5). In men, gonorrhea primarily manifests as acute urethritis characterized by a purulent discharge. It is often accompanied by dysuria but without frequency or urgency. Incubation periods range from 1-10 days, though symptoms typically appear within 2-5 days. Although most infections in men are clinically overt, a small proportion remain asymptomatic. Complications of gonorrhea in men include epididymitis, penile edema, lymphangitis, periurethral abscesses, prostatitis, seminal vesiculitis and infections of the Tyson's and Cowper's glands (6).

In women, the primary site of gonococcal infection is the endocervix, although *N. gonorrhoeae* may be isolated from the urethra, rectum, Skene's and Bartholin's glands. Incubation periods in women are not well established, although symptoms usually occur within 10 days of infection. Symptoms of gonococcal infections in women include cervicitis that manifests as increased vaginal discharge, urethritis, dysuria (usually without frequency or urgency), and intermenstrual bleeding. A frequent complication of gonococcal infection in women is pelvic inflammatory disease (6). Other complications of gonococcal infections include perihepatitis (the result of spillage from the fallopian tubes and spread through the peritoneal cavity), anorectal, and pharyngeal gonorrhea.

According to some reports as many as 90% of women, are asymptomatic and have subclinical infection. Coinfections with *Chlamydia trachomatis* and *Trichomonas vaginalis* are also common (6).

In rare cases, gonococcal bacteremia may result in disseminated gonococcal infections characterized by septic arthritis, polyarthrititis and dermatitis. Newborns may contract gonococcal infections during passage through the birth canal. Gonococcal infections in infants typically manifest as conjunctivitis, known as ophthalmia neonatorum.

Laboratory diagnosis of gonococcal infections

Samples are usually collected using rayon, Dacron or calcium alginate tipped swabs on a plastic or wire shaft (cotton swabs are unsuitable because fatty acids that are present in cotton may inhibit the growth of gonococci). Swabs are inserted between 1 – 3 cm into the endocervical canal or the male urethra to collect specimens. If transport is required, Amies charcoal media or Jembec chambers with modified New York City or Thayer-Martin media may be used. Nowadays, several commercial transport media are available which support better survival of *Ng* (7, 8). For males, Gram stained smears of the specimen are diagnostic if neutrophils or polymorphonuclear leukocytes (PMNs) with intracellular Gram-negative *Ng* are observed (sensitivity > 99%, specificity >95%). For females, the endocervical canal is colonized with non-pathogenic *Neisseria* spp. and Gram staining is not specific. Additionally, pharyngeal and rectal specimens are also unsuitable for diagnosis by Gram's stain because of the presence of *N. meningitidis* and other commensal *Neisseriae*. Sterile specimens are cultured on chocolate agar, while non-sterile specimens are cultured on Thayer-Martin, or Martin-Lewis medium (9) at 37 °C with 5% CO₂ (10). *Ng* have to be biochemically distinguished from other Gram-negative oxidase-positive cocci. Most other species of *Neisseria* ferment both glucose and maltose (*N. meningitidis*, *N. lactamica*, *N. polysaccharea*, *N. subflava*, *N. sicca*, *N. mucosa*), or neither (*N. cinerea*, *N. flavescens*, *N. elongata*). *Moraxella catarrhalis* are also oxidase-positive, Gram-negative cocci, but do not ferment

any carbohydrates used for standard biochemical diagnosis. On the basis of standard biochemical reactions *Ng* may be confused with *Kingella denitrificans* (Gram-negative coccobacilli) since they both ferment only glucose and no other carbohydrates; but they are readily distinguished from the latter, which cannot reduce nitrates (11). Nowadays, most clinical centers diagnose gonorrhea using one of the FDA approved nucleic acid amplification tests (NAATs). These tests have high sensitivity and specificity and their test performance characteristics on urine are similar to samples obtained from the genital tract (12-15). Further, the samples are stable at room temperature for long periods, which facilitates transportation. The ease of sample collection and processing makes NAATs the test of choice for screening of large populations. NAATs, however, do not permit detection of drug resistant strains, which requires culture. Antibiotic susceptibility assays are usually performed using the modified Kirby-Bauer disk diffusion method or E-test (not approved for cefixime) using *Ng* ATCC 43069 as a control strain (16).

Pathogenesis of gonorrhea

The pathogenesis of *Ng* remains poorly understood. It is thought that in women, *Ng* get opsonized with the complement (C') protein C3 fragments (C3b and iC3b) within the cervix (17). The C3b/iC3b then mediates entry into the cervical epithelia via complement receptor 3 (CR3; CD11b/CD18) that is present on primary cervical epithelial cells (18). Entry is facilitated by formation of ezrin and vinculin-rich focal complexes followed by membrane ruffling (19). Following their entry into cervical epithelial cells, gonococcal phospholipase D (*Ng*PLD) upregulates CR3 expression, which allows further infection (20). The exact role of *Ng*PLD remains yet to be determined. The expression of CR3 gradually decreases with ascension in the female genital tract. Concomitantly, the expression of lutropin receptor (LHr) increases (21) which recognize the gonococcal moonlighting protein L12 (22, 23). It is thought that *Ng* infects the epithelium of the female upper genital tract via LHr receptors (24). In the upper tract, *Ng* interacts with non-ciliated tubal epithelium although only ciliated epithelial cells are shed (24). Gonococcal lipooligosaccharide (LOS) (25-29) and peptidoglycan (PG) (30) also have a cytotoxic effect on tubal epithelium, which leads to a surge in pro-inflammatory cytokines such as TNF- α (29). Some, but not all, studies report the presence of TNF- α , IL-1 β , IL-6, and IL-8 in endocervical secretions (31).

In males, the gonococcal LOS interacts with the asialoglycoprotein receptor (ASGP-R) on urethral epithelial cells (32). Data from clinical specimens as well as primary cell cultures reveal that endocytosis of *Ng* occurs via actin- (33) and clathrin- (34) dependent processes subsequent to pedestal formation secondary to *Ng*-ASGP-R interactions (34). *Ng* are then transcytosed across the cell leading to the spread of infection. LOS also triggers the secretion of TNF- α , IL-1 β , IL-6, and IL-8 from the urethral epithelium. Chemokine and cytokine release leads to the influx of a large number of a large number of PMNs which is the pathognomonic feature of gonorrhea (31).

In the absence of opsonins, gonococci not only survive and multiply within PMNs, but also induce inflammatory responses which exacerbate the pathology (22, 35). In order to survive the oxidative burst inside PMNs, gonococci elaborate several virulence factors such as catalase, cytochrome oxidase, superoxide dismutase, manganese transport systems, DNA and protein repair systems including MutY, RecA, Uvr and MsrAB, and the metalloproteinase NGO1686. Gonococci can also resist non-oxidative killing using factors such as multiple transferable resistance system (MtrCDE) and fatty acid resistance system (FarAB) efflux pumps, MisRS, and peptidoglycan O- acyltransferase (PacA) (22). The gonococcal porin (Por) also inhibits phagocytosis and subsequent killing via diverse mechanisms (31). Inside neutrophils, the gonococcal LOS becomes sialylated and must be desialylated in order to enable invasion of male urethral epithelial cells but not the female cervical epithelia. Neuraminidases present on spermatozoa, cervical

epithelial cells, neutrophils, and macrophages de-sialylate gonococci, which facilitates disease transmission to males. It is thought that desialylated gonococci attach to the ASGP-R on surface of spermatozoa during transmission from males to females (31, 36).

Antibiotic resistance in Neisseria gonorrhoeae

Neisseria gonorrhoeae has become extremely drug resistant. A case-report from 2014 describes a multidrug resistant *Ng* isolated in the UK, but acquired in Japan. In this instance, *Ng* had acquired resistance to combination therapy comprising ceftriaxone (a third-generation cephalosporin) and azithromycin (a macrolide) (37). A combination of ceftriaxone and azithromycin is currently the CDC recommended treatment regimen for *Ng* (38).

Before the 1930s *Ng* infections were treated with potassium permanganate (KMnO₄), silver compounds, herbs and hypothermia (39). With the advent of antibiotics, *Ng* was amenable to treatment with sulfonamides (40) and penicillin (41). However, by the mid-1940s *Ng* had acquired resistance to sulfonamides (42), and by the late-1940s resistance to penicillin (introduced in mid-1940s) had begun to emerge (43-47). By the 1960s treatments with spectinomycin and tetracyclines had become commonplace. However, the efficacy of these antibiotics too, was short-lived; spectinomycin resistance was reported in the late 1960s (48) and tetracycline resistance was observed in the early to mid-1980s (49). Between 1990 and 2000, strains of *Ng* were reported that were resistant to fluoroquinolones (50-52), macrolides (53-59), and some third generation cephalosporins (cefixime) (60-62). High-level resistance to ceftriaxone was first reported in 2010 (63). Subsequently, treatment failures with ceftriaxone have been reported from Japan, Europe, UK, Canada, Australia, and South Africa. It is

predicted by some experts that by 2020 circulating strains of *Ng* will be untreatable using conventional antibiotics (37, 64, 65).

Development of vaccines against gonorrhea

Currently, there are no safe and effective vaccines against gonorrhea. Multiple factors have stymied the development of vaccines directed against *Ng*. The primary reason has been the lack of a conserved broadly protective antigen (Ag) for targeting. Most targetable *Ng* Ag(s) are variably expressed across strains, and those that are expressed, undergo widespread Ag variation. Moreover, the correlates of protection against gonorrhea remain unknown. Finally, the lack of a reliable small animal model has also hindered vaccine development.

Only two vaccine candidates, purified pilin (Pil) and killed whole cells, have undergone human clinical trials, and unfortunately, both failed to confer protection against infection (66). The purified Pil-based vaccine comprising PilE (fibril) and low amounts of PilC (minor pilus associated protein) elicited a good antigenic response with the production of antibodies (Ab[s]) that were bactericidal *in vitro*. However, Pil is a highly variable Ag and the vaccine failed to confer protection in humans challenged with *Ng* strains expressing heterologous Pil. The crude killed whole cell vaccine trial conducted in a Inuit population in Northern Canada was well tolerated, but also not protective (67).

Several gonococcal Ag(s) have been reported to be immunogenic, but only three have demonstrated efficacy in the mouse vaginal infection/colonization model (68). In female mice immunized (intra-nasally) with outer membrane (OM) preparations, *Ng* specific immunoglobulin (Ig) G(s) and IgA(s) reactive with

multiple targets were identified from vaginal washings. Serum from immunized mice were also shown to be bactericidal. However, the identity of the targets were not characterized (69). In another study, replicating viruses were employed to immunize mice with refolded recombinant Por B (68). Another candidate vaccine is a peptide mimic ('mimitope') of a LOS epitope that is expressed broadly and is important for virulence (70). This LOS epitope forms the basis of this thesis and has been described later.

Lipooligosaccharide — a ubiquitous and immunogenic virulence factor

LOS, or lipopolysaccharide (LPS) is the most abundant component of Gram-negative OM (Fig. 2). Both the LOS and the LPS have a hydrophobic lipid A and a core oligosaccharide (OS) comprising 2-Keto-3-Deoxy-D-*manno*-octonates (KDO), heptoses (Hep), and hexoses (Hex; usually 6 or less) extending from the Hep residues (Fig. 3). While LOS contains only the core OSs, LPS is characterized by repeating O-linked glycans extending from the core OS (Fig. 3) (71).

LOS is also an important gonococcal virulence factor. It mediates attachment to the ASGP-R on spermatozoa and epithelial cells to facilitate transmission and local spread of infection, respectively (31). Moreover, amounts of LOS as little as 0.015 µg/mL impair ciliary activity and lead to necrosis of the tubal epithelium (28, 72). Furthermore, LOS lipid A and heptose-1,7-*bis*phosphate can lead to NF-κB activation via TLR4-MyD88 (73, 74) or TIFA dependent (75) pathways respectively. Activation of NF-κB results in synthesis of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 (76). This cytokine storm causes the influx of the large number of PMNs, a hallmark of gonococcal infections. A majority of gonococcal infections however are asymptomatic, especially in women. Gonococcal LOS can upregulate Type I interferons (IFN) such as IFN-β via the TLR4 – TRIF-dependent pathway (73). IFN-β suppresses gonococcal killing and may promote a carrier like state (73).

C' is an important anti-*Neisserial* defense mechanism (77-79). TNF- α , IL-1, and IL-6 upregulate synthesis of acute phase mediators such as C4-binding protein (C4BP) (80). Some strains of *Ng* can bind C4BP via their Por(s) and resist the deleterious effects of the classical C' pathway (81). Acquisition of the ability to resist to complement enhances virulence of *Ng*. *Ng* can scavenge CMP-Neu5Ac from the host to sialylate its LOS, which enables *Ng* to bind factor H (FH) which makes them resistant to the alternate pathway of C' (82). Sialylation may also decrease binding of IgG, thereby inhibiting the classical pathway as well (83).

The gonococcal LOS is also immunogenic. Several anti-LOS antibodies (Ab[s]) have been isolated from normal human serum (NHS). These Ab(s) have been described to bind different epitopes including the outer OS(s) as well as the buried KDO(s) (84). Furthermore, some of the Ab(s) activated the classical C' pathway and were bactericidal (85). Given its ubiquitous distribution, its critical role in pathogenesis and its immunogenicity, LOS represents an attractive target for a vaccine or an antibody based therapeutic.

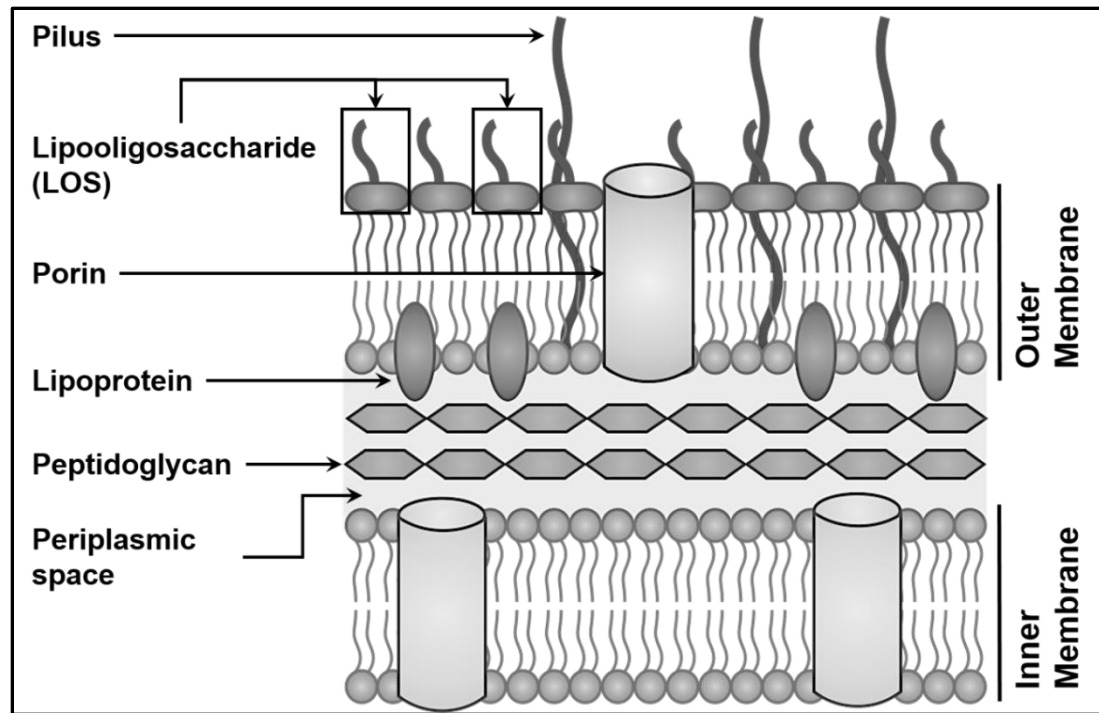


Figure 2. The Gram-negative cell membrane. The Gram-negative cell membrane comprises two phospholipid bilayers with a layer of peptidoglycan in-between. Proteins are embedded in both the outer and the inner membranes. The lipooligosaccharide (LOS) or lipopolysaccharide (LPS) is present in the outer membrane facing the extra-cellular milieu. The LOS or LPS (boxed) is the most abundant molecule in the Gram-negative outer membrane.

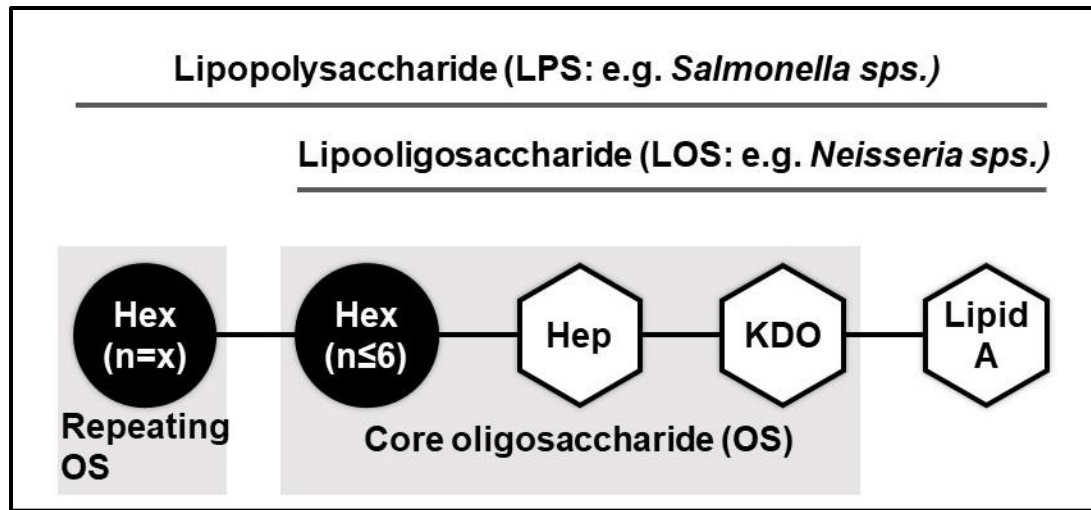


Figure 3. Schematic representation of the LOS and LPS. The Gram-negative LPS molecule has three parts; (i) Lipid A which is hydrophobic and anchors LPS to the outer membrane, (ii) the core oligosaccharide (OS) comprising the 2-Keto-3-Deoxy-D-manno-octonic acid (KDO) residues, two Heptoses (Hep), and up to six Hexose (Hex) residues, and (iii) and the repeating O-linked Hex(s). LPS is usually expressed by members of the Enterobacteriaceae family such as *Salmonella* sps., *Shigella* sps., *Klebsiella* sps., and other Gram-negative organisms such as *Pseudomonas* sps. In organisms such as *Neisseria* sps. and *Haemophilus* sps. that are not normally exposed to biliary acids, the repeating O-linked Hex(s) are absent; only the Lipid A and the core OS is present; this molecule is called the lipooligosaccharide or LOS.

Ng LOS is targeted by monoclonal antibody (mAb) 2C7

In the 1990s several mAb(s) that reacted with the gonococcal LOS, including mAb 2C7, were generated. mAb 2C7 (IgG3 λ) was originally obtained from splenic B-cells of mice immunized with outer membrane vesicle preparations from the *Ng* strain WG, specifically to characterize LOS. Subsequently, the B-cells were fused to the murine myeloma cell line Sp2/0-Ag14 to generate stable hybridomas (86). In a study conducted in Boston, >94% of clinical isolates reacted with this antibody (87). In more recent studies conducted by our group using minimally passaged isolates from a STI clinic in Nanjing, China, 100% of the isolates reacted with this mAb (Sunita Gulati – personal communication). O-deacylated LOS was acid hydrolyzed to liberate lipid A, and the remaining OS component was analyzed by mass spectrometry. Subsequently, the mAb 2C7 epitope was determined to consist of a β -linked lactose from HepI (Gal- β 1,4-Glc- β 1,4-HepI) and an α -linked lactose from HepII (Gal- β 1,4-Glc- α 1,3-HepII) (88).

The LOS epitope recognized by mAb 2C7 appears not only to be widely expressed, but is also important for pathogenesis. In a murine infection model of vaginal colonization, knocking out the 2C7 epitope attenuates *Ng* infection (70). Furthermore, it has been observed that serial passage of laboratory *Ng* strains that initially poorly colonize mouse vaginal tracts results in progressively exalted infection *in vivo*. Whole genome sequencing established increased expression of the mAb 2C7 epitope as a result of *lgtG* becoming phase-varied 'ON', and

constitute a probable explanation for enhanced bacterial infectivity in mice (89). Therefore, the mAb 2C7 epitope within the *Ng* LOS forms an excellent target for immunotherapeutic and vaccine Ab(s).

In the murine vaginal colonization model, passive immunization with mAb 2C7 shortens the duration and burden of bacterial infection. Furthermore, active immunization with a peptide mimic of the mAb 2C7 epitope also resulted in transient infections and lower bacterial burdens (70, 87). The Ab response to vaccination was specific to the mAb 2C7 epitope because no Ab binding was detected against *Ng* lacking this epitope. The anti-LOS Ab repertoire comprised IgM, IgA, and IgG (IgG1, IgG2a, IgG2b, and IgG3) (70). Thus, mAb 2C7 forms the basis of an anti-gonococcal immunotherapeutic, while a peptide mimic of its epitope is being developed as a candidate vaccine.

mAb 2C7 activates the classical complement cascade

The correlate(s) of protection against gonococcal infections remain to be fully elucidated. However, C' mediated defenses are believed to play a critical role against gonorrhea for the following reasons. First, C' deficiencies are associated with recurrent *Neisserial* infections (77, 79, 90). Second, C'-dependent serum bactericidal activity mediated by immune Ab is the correlate of protection against the closely related pathogen *Neisseria meningitidis* (91). Thus, although the mechanism whereby mAb 2C7 attenuates gonococcal infection in the experimental murine model is unknown, work thus far has focused on its ability to activate the classical C' pathway and kill *Ng* in C'-dependent serum bactericidal assays.

Antibodies activate the classical C' cascade (Fig. 4) via their Fc regions. Human IgG subclasses differ in their ability to activate C'. In general, human IgGs activate C' in the order: IgG3>IgG1>IgG2, while IgG4 does not activate C'. In contrast to IgG, where the cooperation between six molecules is required to activate C' (92), a single IgM molecule can activate C'. This is because IgM is polymer (pentameric or hexameric in the presence or absence of the J chain, respectively) and each target-bound IgM can bind to a C1 complex (93, 94). Thus, on a molar basis, IgM is the most potent activator of the classical pathway.

The classical pathway is usually initiated by binding of antibodies to their target antigens. Binding of an antibody to its target exposes a binding site for the

trimolecular C1 complex that comprises a hexameric C1q, which binds Fc, and two molecules each of C1r and C1s. A single globular head of C1q and Fc interact with very low affinity ($K_d \approx 10^{-4}$ M) (95, 96). However, above a certain critical epitope density, Fc domains form hexamers, which then engage each of the six globular heads C1q molecule and results in a high avidity C1q-Fc interaction (97, 98). C1r and C1s are arranged as a tetramer (C1s-C1r-C1r-C1s) and form a Ca^{2+} dependent catalytic subunit (99, 100). Binding of C1q generates a conformational signal that results in auto-activation of C1r, which in turn activates C1s. Some authors suggest intramolecular activation of C1r by C1s is a conformational improbability. Their data suggests C1r activates C1s in a neighboring C1 complex (101). Both molecules are activated through cleavage of an Arg-Ile bond.

Activated C1s cleaves the 77-amino acid C4a fragment from the N-terminus of the α -chain of C4 to form the metastable C4b molecule. This exposes the internal thioester bond of C4b (102) that can react readily with nucleophilic (*i.e.*, electron-donating groups) groups such as $-\text{OH}$ or $-\text{NH}_2$ on surfaces to form covalent ester or amide bonds, respectively (103). If the nascent carbonyl group in the thioester moiety does not interact with a surface, it will react with water and remain in solution. There are two isoforms of C4 expressed by most humans, called C4A and C4B (104), which dictates the type of bond formed by C4b (note that C4a and C4b represent activation products of C4 and are distinct from the C4A and C4B isoforms of intact C4). C4B possesses a His residue at position 1106 in the

α -chain, which imparts to C4B the ability to form ester linkages, while an Asp residue at position 1106 results in 'C4A-like' functionality and preferential amide bond formation (105).

In the next step in classical pathway activation, C2 binds to C4b deposited on a surface. C2 is also cleaved by activated C1s into the C2a fragment, which remains attached noncovalently to C4b and C2b, which is released into solution. C4b2a forms the C3 convertase (C3 cleaving enzyme) of the classical pathway. In this manner, a single C1 complex can cleave several substrate molecules and augment C' activation. Formation of the classical pathway C3 convertase can be inhibited by C4BP. C4BP inhibits the classical pathway by two major mechanisms: i) acts a cofactor in the factor I (FI)–mediated cleavage of C4b to C4c and C4d, and ii) accelerates dissociation of C2a from the classical pathway C3 convertase (C4bC2a), a property called 'decay-accelerating' activity (106-109).

All C' pathways converge at the level of C3. C3 is the most abundant C' component (plasma concentrations range from 1.0 to 1.5 mg/ml) and its fragments serve a variety of functions. C3 fragments deposited on surfaces are opsonins for phagocytes and the anaphylatoxin C3a modulates inflammation, lipid metabolism (C3a-desArg, which is generated by cleavage of the C-terminal Arg by carboxypeptidase N is also called acylation stimulating protein) and tissue regeneration. Similar to C4, the α -chain of C3 also possesses an internal

thioester moiety that forms stable, covalent bonds with target surfaces. Cleavage of the C3a fragment from C3 results in activation of C3 is accompanied by marked structural rearrangements among its various domains. Most notably, the thioester domain that is tucked away in the native molecule becomes completely exposed and capable of reacting with nucleophiles (110). The thioester forms a highly reactive acyl-imidazole intermediate with an extremely short calculated half-life of $\approx 30 \mu\text{s}$ (111). If this reactive group does not bind to a surface $-\text{OH}$ (or in some instances, a $-\text{NH}_2$ group) within this short period, it will react with a water molecule and remain in solution. The high reactivity and short duration of the nascent thioester domain restricts C3 deposition to structures proximate to the site of C3 activation, while sparing more distant (and possibly normal) tissue from unwanted damage.

Binding of an additional C3b molecule to C3 convertase generates the C5 convertase (C4bC2aC3b) that can cleave C5 and initiate the assembly of membrane attack complex (MAC). C5 bears structural homology with C3 and C4, but lacks a thioester domain. The addition of C3b to C3 convertases alters the K_m for C5 >1000 -fold, from far above the physiological concentration of C5 to far below it (112-114). Therefore, situations that favor C' activation and rapid C3b generation also facilitate the generation of cytolytic MAC. Cleavage of C5 results in release of the $\approx 11 \text{ kDa}$ C5a fragment, a key anaphylatoxin with diverse functions. Removal of the C-terminal Arg residue from C5a by carboxypeptidase

N results in formation of C5a-desArg, which retains only 1-10% of the inflammatory activity of C5a (115).

Binding of C5b to hydrophobic sites on cell surfaces exposes binding sites for C6 and C7 to form the C5b-7 complex. Incorporation of C7 confers amphiphilic properties to the assembling MAC and permits direct insertion into cell membranes. C8 then binds to the β -chain of C5b, followed by the addition of one or more C9 molecules. C6, C7, C8 and C9 all belong to the MACPF/CDC (MAC-perforin/cholesterol dependent cytolysin) superfamily of proteins (116, 117), which contain a common set of four core domains; from the N- to C-terminus, a thrombospondin-1 (TSP1) domain, a low-density lipoprotein receptor-associated (LDLRA) domain, a MACPF domain and an epidermal growth factor (EGF) domain. In its fully assembled state, MAC comprises one molecule each of C5b, C6, C7 and C8 and up to 18 molecules of C9. The ring-like membrane configuration of MAC is dependent on C9 polymerization. C9 in its free monomeric native state has a globular conformation (118). Upon polymerization, C9 adopts a tubular structure, where the external aspect of the tubule is hydrophobic and intercalates into membranes, while the inner aspect of the pore is hydrophilic and permits passage of water and ions. While most other pore-forming proteins rely on interactions solely between their MACPF domains, structural studies of poly-C9 revealed unexpected interactions between the TSP1 domain and the MACPF domain of adjacent monomers (119). The TSP1-MACPF interactions permit recruitment of C9 molecules from solution into the nascent

membrane-associated MAC complex. Cryo-electron microscopy analysis of the entire C5b-9 complex revealed a 'split-washer' configuration, as opposed to a symmetric closed-ring conformation seen with perforin and other cholesterol-dependent cytolysins (120). Disruption of the membrane proton motive force during pore formation and osmotic damage mediated by the channel may both contribute to the cytolytic action of MAC. Formation of MAC is thought to be the primary mechanism of action of mAb 2C7.

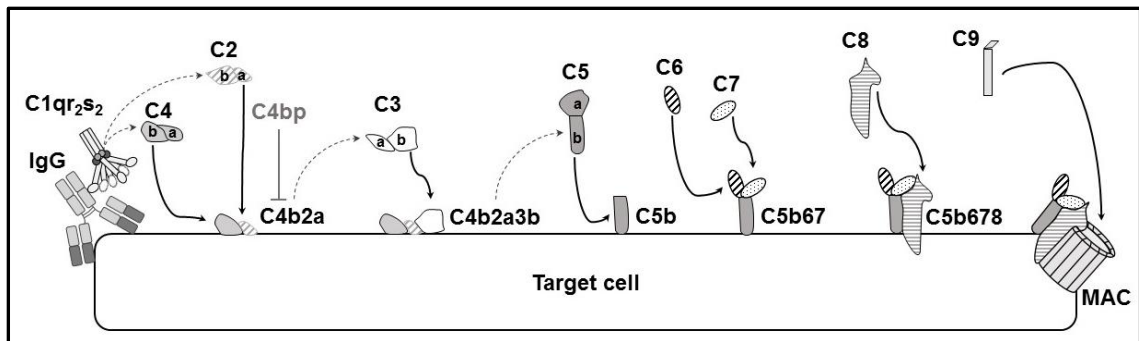


Figure 4. The classical complement (C') cascade. Ab (IgG/IgM) binds to the surface of the target cells/Ag. The globular heads of the hexameric C1q engages amino acid residues in the Fc region of the Ab. The affinity of an individual C1q head for the Fc region is very low, so multiple globular heads of the same C1q molecule must be engaged by multiple Ab(s). This not only enhances the avidity of C1q for the Ag – Ab complex, but also brings about a conformational change such leading to intermolecular or intramolecular activation of C1s by C1r. C1s cleaves C4 into C4a and C4b. C4b gets deposited on the surface of the target forming amide or ester linkages on account of its internal thioester bonds reacting with – NH₂ or – OH groups. C2 which then binds to C4b is also cleaved by C1s into C2a and C2b. C2a remains non-covalently attached to C4b. The C4b2a complex forms the classical pathway C3 convertase which cleaves C3 into C3a and C3b. Binding of C3b to C4b2a generated the classical pathway C5 convertase (C4b2a3b). The C5 convertase cleaves C5 into C5a and C5b. Binding of C5b to hydrophobic surfaces exposes binding sites for C6 and C7 forming the C5b-7 complex. Incorporation of C7 imparts amphiphilic properties to the assembling Membrane Attack Complex (MAC). C8 then binds to the β-chain of C5b followed by the addition of one or more C9. C8 is the first protein of the C' cascade to insert into the membrane. Multiple C9 molecules come together to form a spit washer like pore which leads to the osmotic lysis of cells. The liberated C3a, C4a, and C5a function as anaphylatoxins with diverse functions.

Gaps in knowledge of mAb 2C7 biology

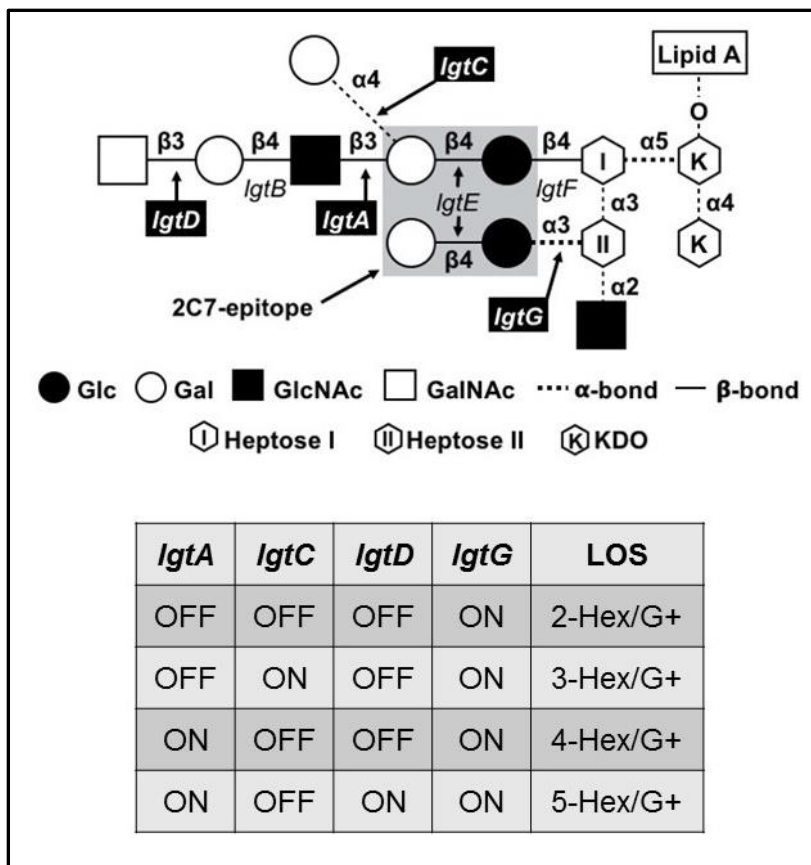
Murine protection experiments with the mAb 2C7 antibody were done with wild type (WT) FA1090 (70). Unpublished observations based on mAb 3F11-reactivity (121) show that the WT FA1090 used in the animal experiments predominantly express the 4-Hex/G+ LOS, or the lacto-N-neotetraose (LNnT) structure. Hexose extensions from gonococcal heptoses, however, are highly variable and *Ng* can express up to eight different LOS structures because of random switching 'ON' and 'OFF' of four phase-variable (pv) *lipooligosaccharide glycosyl transferase* (*lgt*) genes: *lgtA*, *lgtC*, *lgtD*, and *lgtG* (122-124) (Fig. 5). Of these, *lgtG* is 'virtually' 'ON' *in vivo*, resulting in the expression of the mAb 2C7 epitope (87, 89). The basis of pv of the gonococcal LOS is the presence of homo-polymeric poly-G/C tracts within the coding regions of the *lgt* genes, which leads to slipped-strand mispairing (SSM) primarily during DNA replication (123, 124). In SSM, loops are formed during DNA replication (Fig. 6). If the loop is formed in the template strand, it leads to deletion of nucleotides in one of the progeny. If the looping occurs in the nascent strand, it results in addition of nucleotides. Either of these events (addition or deletion of C/Gs) can throw an 'in frame' coding sequence out of frame, or conversely can restore an out of frame coding sequence back in frame. SSM of *lgtA*, *lgtC*, and *lgtD* in the presence of an in-frame *lgtG* (henceforth referred to as G+) results in the expression of at least four distinct LOS structures (2-Hex/G+, 3-Hex/G+, 4-Hex/G+, and 5-Hex/G+), each of which possesses the 'minimal' mAb 2C7 epitope (Fig. 7). Of these, WT FA1090

predominantly expresses a 4-Hex/G+ LOS structure. While all four LOS structures are represented in clinical isolates, the relative importance of each of these structures in pathogenesis is not fully understood. Further, how HcpI glycan extensions (i.e., the structures expressed because of phase variation of *lgtA*, *lgtC* and *lgtD*) affect mAb 2C7 binding and function remains to be determined.

The WT FA1090 that has been used in mAb 2C7 protection studies (70) is a relatively avirulent strain (125). In human experimental urethral infections the infectious dose for this strain has been established to be ~100-fold orders higher than strains such as MS11 (126). MS11, along with eighty percent of circulating *Ng* strains possesses a 57-kb Gonococcal Genetic Island (GGI) (127). Although the function of the GGI is unknown, it is associated with anti-microbial resistance (128). Moreover, it is thought to encode a lytic transglycosylase (*ltgA*) which cleaves PG to form a peptidoglycan-derived cytotoxin (PGCT). Sinha and Rosenthal have established that the most abundant *Ng* PG released into the surrounding milieu is similar to the *Bordetella pertussis* PGCT which is pro-inflammatory and causes IL-1 and IL-6 release (129). Such pro-inflammatory cytokines can enhance *Ng* virulence (as described earlier). WT FA1090 used in mAb 2C7 protection studies (70) also lacks the GGI which may be an important virulence determinant.

Further, WT FA1090 lacks lactoferrin-binding proteins (Lbp) A and B. Thus, it can scavenge iron only from transferrin (Tf). Iron is an important growth factor for *Ng*, and is available from lactoferrin (Lf) as well as transferrin. In experimental human studies where volunteers were challenged with a mixture of equal numbers of WT FA1090 (Tf+Lf-) and a Tf+Lf+ FA1090 mutant, by day 4 or 5, 100% of recovered isolates were the Tf+Lf+ mutant (130). About half of the circulating *Ng* strains have a Tf+Lf- phenotype, while the other half have a Tf+Lf+ phenotype. It is therefore interesting to note that the strain (MS11) shown to have a lower ID₅₀ than FA1090 *in vivo* has a Tf+Lf+ phenotype (126).

Figure 5. Schematic Representation of the Phase-variable (pv) *Ng* LOS. *Ng* expresses a LOS, meaning it has a hexacylated Lipid A O-linked to the core OS comprising two KDO(s), two Hep(s), and Hex extensions emanating from HepI and HepII, but lacks the O-linked repeating sugars. *Ng* can express up to



eight distinct LOS structures. Variation in the *Ng* LOS is due to pv of four *lipooligosaccharide glycosyltransferase (lgt)* genes (indicated within black boxes); *lgtA*, *lgtC*, *lgtD*, and *lgtG*. These genes encode enzymes that catalyze addition of Hex(s) to the Hep(s). Two other *lgt* genes, *lgtF* and *lgtE* are constitutively expressed and responsible for the extension of a lactose adduct from HepI. Thus, this is the LOS structure that *Ng* would express when all four pv genes are 'OFF'. Expression of *lgtG* is almost always 'ON' (>94% clinical samples are mAb 2C7-positive), and this results in the addition of a lactose moiety to HepII. The HepI and HepII lactoses together make up the mAb 2C7 epitope (shaded in gray). Depending on the combinations in which *lgtA*, *lgtC*, or *lgtD* are 'ON' or 'OFF', two, three, four, or five Hex(s) may be extended from HepI. The table denotes the four possible *Ng* LOS structures (in >94% of samples) and their genotypes.

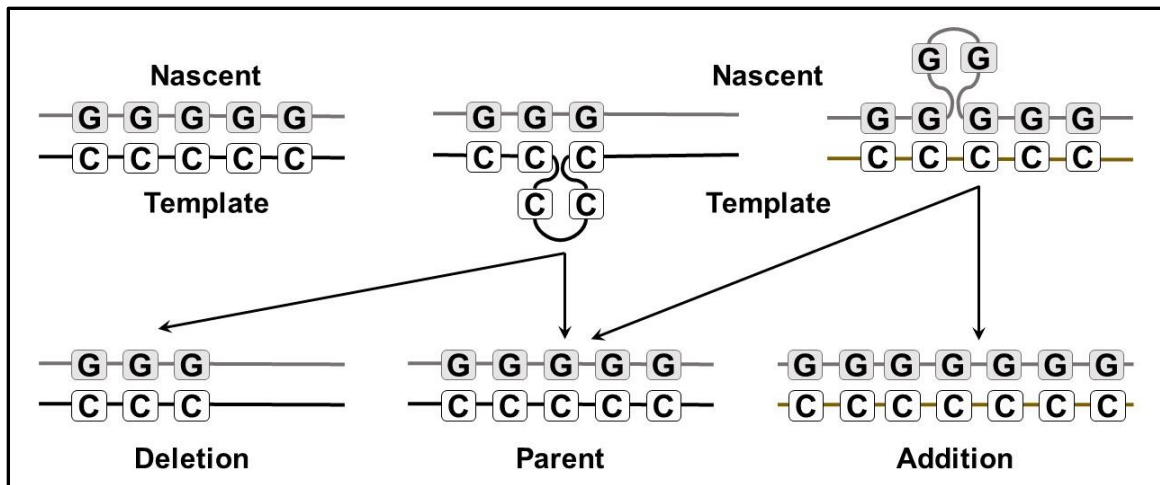


Figure 6. Phase-variation (pv) of the *lgt* genes. As discussed earlier, variation in the *Ng* LOS structure is due to pv in four of the *lgt* genes; *lgtA*, *lgtC*, *lgtD*, and *lgtG*. Presence of homopolymeric poly-G or poly-C tracts within the coding sequence of these genes allow them to be turned 'ON' or 'OFF' during DNA replication by a process known as 'slipped-strand mispairing' (SSM). During DNA replication, the presence of homopolymeric (poly-G/C) tracts may sometimes lead to formation of loops. If the loops form in the nascent (replicating) strand, it leads to addition of nucleotides in one of the two progenies. If, the event occurs in the template strand nucleotides are deleted. Assuming the looping event occurs in one of the two parental DNA strands, one of the progenies maintain the parental DNA sequence. An addition or deletion can throw an 'in frame' coding sequence 'out of frame' or vice-versa thereby regulating the expression of the corresponding enzymes. This culminates in the expression of different LOS structures.

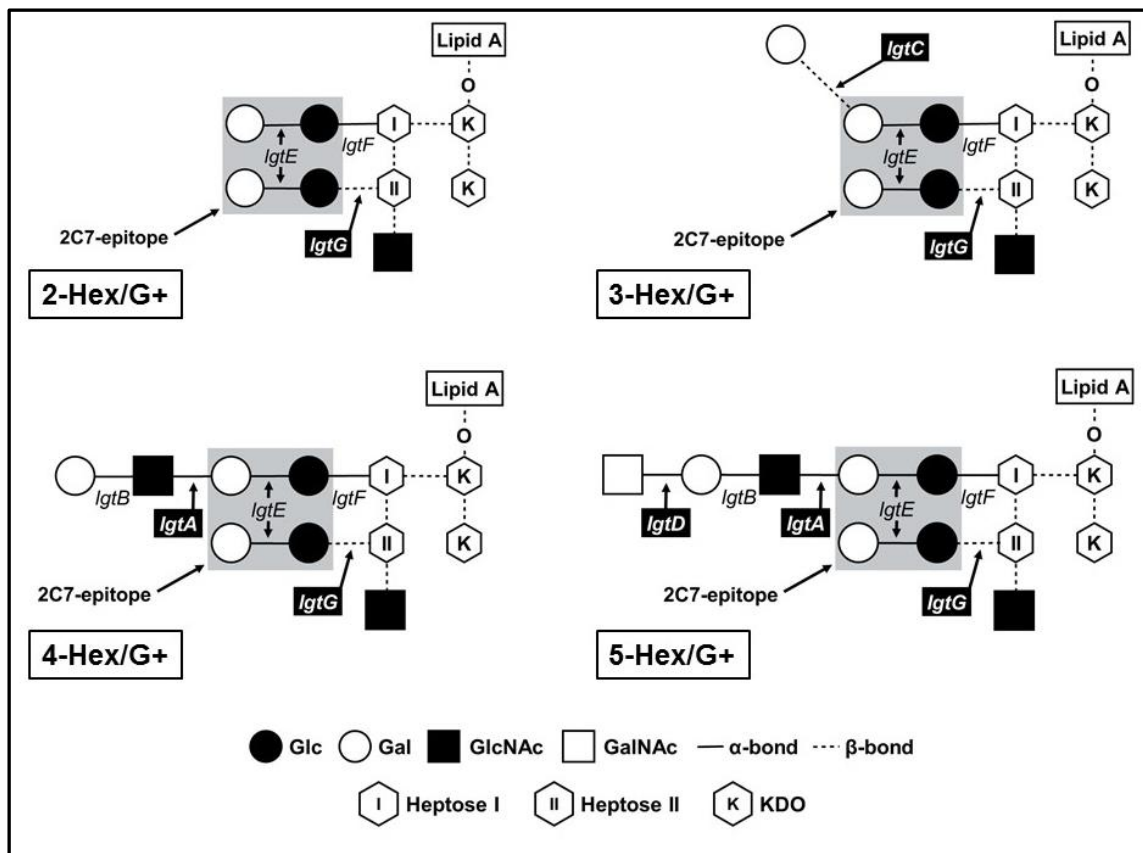


Figure 7. The four LOS structures expressed by >94% clinical isolates of *Ng*. More than 94% of the clinical isolates of *Ng* have the *IgtG* turned 'ON' (G+) thereby expressing the HepII lactose which makes them mAb 2C7 reactive. Depending on the combination in which the other three pv genes are turned on or off, *Ng* can express four different LOS structures: 2-Hex/G+, 3-Hex/G+, 4-Hex/G+, and 5-Hex/G+ (clockwise from top left). Most *Ng* express more than one LOS structures, although the proportion in which they do so *in vivo* is variable.

Scope and specific aims of this dissertation

Will a murine mAb 2C7 epitope-based vaccine be effective against all four distinct *Ng* LOS structures that can theoretically be recognized by the Ab? Is murine mAb 2C7 effective *in vitro* against more virulent *Ng* strains with lower infectious doses (such as MS11)? Do HcpI glycan structures impact infectivity in the mouse vaginal colonization model? These were the major unanswered questions surrounding the efficacy of a mAb 2C7-epitope based vaccine that this dissertation sought to shed light on.

In light of data obtained over the course of the above studies, I also proceeded to ask whether murine mAb 2C7 could be developed as a therapeutic antibody against multidrug-resistant *N. gonorrhoeae*. And, would such a therapeutic be effective *in vitro* and *in vivo* against all 2C7-positive LOS glycoforms? As a first step to answer these questions, a chimeric mAb 2C7 (2C7-Ximab) was created in collaboration with Genmab B.V. The Fc region of 2C7-Ximab was further modified to enhance its C' activating properties. Thus, I also asked whether this modification conferred any advantage over a chimeric with a native (WT) Fc?

The specific aims of this dissertation were:

1. To determine the effects of pv of the gonococcal LOS on the *in vitro* efficacy of a 2C7-epitope based vaccine.
 - 1.1. To create a set of isogenic mutants with defined LOS structures in a more virulent *Ng* strain such as MS11.
 - 1.2. To test the *in vitro* efficacy of murine mAb 2C7 against different LOS mutants created in this background.
2. To determine the effectiveness of a chimeric mAb 2C7 (2C7-Ximab) engineered to enhance C' activation *in vitro* and *in vivo*, and to investigate the role of pv of the gonococcal LOS in pathogenesis.
 - 2.1. To compare *in vitro* efficacies of two 2C7-Ximab constructs that differed in their ability to activate complement against the *Ng* LOS mutants.
 - 2.2. To test the effect of pv of the gonococcal LOS on *Ng* pathogenesis *in vivo*.
 - 2.3. To test the *in vivo* efficacy of the more potent (based on *in vitro* studies) 2C7-Ximab against the four 2C7-positive *Ng* LOS mutants.

CHAPTER II

Phase-variable heptose I glycan extensions modulate efficacy of 2C7 vaccine antibody directed against *Neisseria gonorrhoeae* lipooligosaccharide

(Originally published in The Journal of Immunology. Chakraborti, S., L. A. Lewis, A. D. Cox, F. St Michael, J. Li, P. A. Rice, and S. Ram. 2016. Phase-Variable Heptose I Glycan Extensions Modulate Efficacy of 2C7 Vaccine Antibody Directed against *Neisseria gonorrhoeae* Lipooligosaccharide. *J Immunol* 196: 4576-4586. © [2016] The American Association of Immunologists, Inc.)

Abstract

Neisseria gonorrhoeae, the causative agent of the sexually transmitted infection, gonorrhea, has developed resistance to most conventional antibiotics. Safe and effective vaccines against gonorrhea are needed urgently. A candidate vaccine that targets a lipooligosaccharide (LOS) epitope recognized by monoclonal (mAb) 2C7 attenuates gonococcal burden in the mouse vaginal colonization model. Glycan extensions from the LOS core heptoses (Hepl and HepII) are controlled by phase-variable LOS glycosyltransferase (*lgt*) genes; we sought to define how Hepl glycan extensions affect mAb 2C7 function. Isogenic gonococcal mutants in which the *lgt* required for mAb 2C7 reactivity (*lgtG*) was genetically locked 'ON' and the *lgt* loci required for Hepl variation (*lgtA*, *lgtC* and *lgtD*) were genetically locked 'ON' or 'OFF' in different combinations were created. We observed 100% complement-dependent killing by mAb 2C7 of a mutant that expressed lactose (Gal-Glc) from Hepl, while a mutant that expressed Gal-Gal-Glc-Hepl fully resisted killing (>100% survival). Mutants that elaborated 4- (Gal-GlcNAc-Gal-Glc-Hepl) and 5-glycan (GalNAc-Gal-GlcNAc-Gal-Glc-Hepl) structures displayed 'intermediate' phenotypes (<50% killing with 2 µg/mL and >95% killing with 4 µg/mL of mAb 2C7). The contrasting phenotypes of the lactose-Hepl and the Gal-Gal-Glc-Hepl LOS structures were recapitulated with phase-variants of a recently isolated clinical strain. Despite lack of killing of the Gal-Gal-Glc-Hepl mutants, mAb 2C7 deposited sufficient C3 on these bacteria for opsonophagocytic killing by human neutrophils. In conclusion, mAb

2C7 showed functional activity against all gonococcal HcpI LOS structures defined by various *IgtA/C/D* 'ON/OFF' combinations, thereby providing further impetus for use of the 2C7 epitope in a gonococcal vaccine.

Introduction

Gonorrhea, caused by the gram-negative diplococcus *Neisseria gonorrhoeae* (the gonococcus) is the most common bacterial sexually transmitted infection (STI) worldwide (2nd most common in the US). While most cases result in 'uncomplicated' infections of the lower genital tract (urethritis in men and cervicitis in women), gonorrhea may sometimes lead to complications such as pelvic inflammatory disease and disseminated gonococcal infection. Serious sequelae of gonorrhea include infertility and ectopic pregnancy. Infected individuals who are asymptomatic or minimally symptomatic constitute an important reservoir for the transmission of infection.

Globally, about 78 million new cases of gonorrhea occur annually (131). As a result of the emergence of antibiotic resistant strains, including strains resistant to third-generation cephalosporins such as cefixime and ceftriaxone (39) and the lack of vaccines (67, 132) or novel anti-infective therapeutics, gonorrhea has become a major public health concern. A safe and effective vaccine would be a key step in curbing the spread of multidrug-resistant gonorrhea.

An obstacle to gonococcal vaccine development is the wide antigenic variation and/or variable expression of antigens that may elicit a protective response (e.g., pilin, opacity proteins, porin (Por) B, lipooligosaccharides [LOS(s)]) (67, 132, 133). In addition, certain conserved antigens elicit non-protective, and in some

instances subversive responses; an example of the latter is Reduction modifiable protein (Rmp) (134).

Despite its phase-variable nature (135), gonococcal LOS has been considered as a potential vaccine antigen (85, 136). Men who were experimentally infected with *N. gonorrhoeae* were less likely to become infected upon re-challenge if they elicited an anti-LOS IgG response following the initial infection (137). Previous work by our group identified an epitope on gonococcal LOS that is recognized by a monoclonal antibody (mAb) called 2C7 (and therefore referred to as the '2C7 epitope) and was expressed on 94% of gonococci (64 out of 68) recovered directly from human cervical secretions (138). Gonococcal infection in humans elicits an antibody response against the 2C7 epitope (138). Expression of a lactose residue from heptose (Hep) II is required for binding of mAb 2C7 (139). Addition of an α -linked Glc residue at the 3-position of HepII represents the first step in synthesis of the lactose extension from HepII and is mediated by the phase-variable LOS glycosyltransferase G (*lgtG*) (140).

Expression of *lgtG* is important for murine infection (70). Passive administration of mAb 2C7, as well as active immunization with a peptide mimic (mimitope) of the 2C7 epitope that was configured as a 'multi-antigen peptide' on a poly-lysine 'backbone' significantly shortened the duration and burden of infection in the murine vaginal colonization model of gonorrhea (70). Taken together, these data

suggest that the 2C7 epitope represents a promising gonococcal vaccine candidate.

Phase variation of LOS glycan extensions is mediated by slipped-strand mispairing at homopolymeric tracts within the coding regions of the *lgt* genes; *lgtA*, *lgtC*, *lgtD* modify glycan extensions from HepI; *lgtG* permits glycan extensions from HepII, as discussed above. Phase variation permits gonococci to express several distinct LOS structures that differ in their glycan composition (135, 141). Modulation of mAb 2C7 function by variations in HepI glycans has not been studied, is an important consideration that may impact the efficacy of a 2C7 epitope-based vaccine and forms the basis of this study.

Materials and Methods

Bacterial strains and culture conditions

The *Neisserial* strains used in this study are described in Table 1. *N. gonorrhoeae* MS11 4/3/1 is a variant of MS11 VD300 with an IPTG inducible *pilE* that controls pilus expression (142). UMNJ60_06UM was recovered in 2013 from a symptomatic male with urethritis in Nanjing, PRC (143), and shows intermediate resistance to ceftriaxone (Etest MIC = 0.38 µg/mL and disc = 35 mm (sensitive ≥ 35mm). UMNJ60_06UM belongs to NG-MAST sequence type (ST) 3289 and MLST ST 1600.

Gonococcal strains were routinely cultured at 37°C in an atmosphere of 5% CO₂ on chocolate agar enriched with a chemically defined supplement (termed Isovitalex) used as an additive for cultivation of nutritionally fastidious microorganisms. For growth in liquid culture Morse A supplemented with Morse B and isovitalex were used (144). When used, antibiotics were added to GC agar plates at the following concentrations: erythromycin (Erm) 5 µg/mL, kanamycin (Kan) 100 µg/mL and streptomycin (Sm) 10 mg/mL. To induce pilus expression and enable transformation, strain MS11 4/3/1 was cultured on GC agar plates supplemented with 0.25 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

E. coli Top10, XL-10 gold and INVαF' (Invitrogen) were cultured on LB agar supplemented, as needed, with antibiotics at the following concentrations;

ampicillin (125 µg/mL), Kan (50 µg/mL), Erm (400 µg/mL) or Sm (50 µg/mL).

INVαF', a naturally streptomycin sensitive strain, was used for propagation of all plasmids containing the Erm^R-Sm^S streptomycin sensitivity cassette.

Construction of mutants

We created eight LOS mutants in MS11 4/3/1 (Table 1), in which expression of the four phase-variable *lgt* genes (*lgtG*, *lgtA*, *lgtC* and *lgtD* [shown schematically in Fig. 1A]) was genetically fixed either 'ON' or 'OFF' (or deleted). *lgtG* was insertionally inactivated (G- mutants; HepII unsubstituted) by amplifying *lgtG::kan* from FA19 *lpt6A lptA lgtG* (kindly provided by Dr. William Shafer, Emory University) using *lgtG_F* and *lgtG_R* primers (**Supplemental Table S1**), and subsequently transforming MS11 4/3/1 with the purified PCR product. The kanamycin marker in *lgtG* in FA19 was derived from pCK49 (145). Inactivation of *lgtG* in kanamycin resistant MS11 transformants was confirmed by PCR and DNA sequencing.

lgtG was fixed 'ON' (G+ mutants; HepII substituted with lactose) by first exchanging the wild type *lgtG* with *lgtG* containing the *ermC'-rpsL_{F62}* cassette (pRYGW2ES1; Supplemental Table S2) that encodes resistance to Erm and sensitivity to Sm (146). Erm-resistant transformants were subsequently transformed with an *lgtG* - 'ON' construct (p*lgtG*+) (Supplemental Table S2) in which the C₁₁ homopolymer had been changed to the non-phase variable

sequence CCCCTCCGCCA. *lgtG*-‘ON’ (G+) mutants were selected for resistance to streptomycin and screened for sensitivity to erythromycin (146).

HepI glycan mutants were made in both the MS11 G+ and G- backgrounds, by first exchanging each of the three phase variable HepI *lgt* genes (*lgtA*, *lgtC*, *lgtD*) with an *ermC*'-*rpsL*_{F62} cassette (plgtA-ES, plgtC-ES, plgtD-ES; Supplemental Table S2), followed by transformation with the respective locked ‘ON’ (plgtA-‘ON’, plgtC-‘ON’, plgtD-‘ON’; Supplemental Table S2), locked ‘OFF’ (plgtC-‘OFF’; Supplemental Table S2) or mutated (segment deleted) form of each gene (plgtA-del and plgtD-del; Supplemental Table S2).

To insert the *ermC*'-*rpsL*_{F62} cassette into each *lgt*, the homopolymeric phase variation sequence in each *lgt* was deleted and a SmaI restriction site was incorporated by overlap extension PCR (using the respective F-Ext / R-Int and F-Int / R-Ext primers; Supplemental Table S1). Each mutated (homopolymer deleted and SmaI incorporated) *lgt* was amplified (F-Ext and R-Ext; Supplemental Table S1) and cloned (separately) into pCR2.1 TOPO TA (Invitrogen, USA). The *ermC*'-*rpsL*_{F62} cassette was extracted from pFLOB4300 (provided by Dr. Janne G. Cannon, University of North Carolina, Chapel Hill) with PvuII and inserted into the SmaI site of each phase variable HepI *lgt* gene (See plasmids plgtA-ES, plgtC-ES and plgtD-ES; Supplemental Table S2). Plasmids carrying *ermC*'-*rpsL*_{F62} were maintained in the streptomycin sensitive *E. coli* INVαF' (Life Technologies, USA).

Wildtype *lgtA*, *lgtC* and *lgtD* were amplified from MS11 4/3/1 chromosomal DNA by PCR using the corresponding F-Ext and R-Ext primers (Supplemental Table S1) and the amplicons ligated with pCR2.1 TOPO TA cloning vector (Life Technologies, USA) and transformed into chemically competent *E. coli* TOP10 (Life Technologies, USA) per the manufacturer's instructions (Supplemental Table S2). Plasmids with *lgtA*, *lgtC* and *lgtD* locked 'ON' and *lgtC* locked 'OFF' were generated using Quick Change Lightning Multi Site-Directed Mutagenesis kit (Agilent Technologies, USA) with the corresponding mutagenic primers (Supplemental Table S1) and transformed into chemically competent XL-10 Gold *E. coli* cells as per manufacturer's recommendations (Supplemental Table S2). Double digestion and subsequent ligation of plasmids with wild type *lgtA* and *lgtD* with BbsI and SspI, and NotI and SpeI respectively, yielded plasmids with deletion mutations in *lgtA* and *lgtD* (Supplemental Table S2).

Replacement of the *lgtC*-locked 'ON' gene in the mutant that expressed the 3-Hex HepI/*lgtG*⁺ LOS structure with *lgtC* locked- 'OFF' yielded the 2-Hex-HepI/*lgtG*⁺ mutant. Conversely, locking *lgtC* 'ON' in the 2-Hex-HepI/*lgtG*⁺ mutant yielded a *lgtG*⁺ mutant that expressed 3-Hex from HepI.

UMNJ60_06UM *lgtA::kan* was constructed as previously described (147).

Inactivation of *lgtA* was confirmed by PCR and western blot using mAb 3F11 (mAb 3F11 described below). UMNJ60_06UM *lgtA::kan* 2-Hex and

UMNJ60_06UM *lgtA::kan* 3-Hex were identified by western blot; UMNJ60_06UM

IgtA::kan 2-Hex reacted with mAb L8 but not mAb L1 (both mAbs are described below) and UMNJ60_06UM IgtA::kan 3-Hex reacted with mAb L1 (recognizes the globotriose Gal α 1,4-Gal β 1,4-Glc structure, also called the P^K-like structure) but not mAb L8 (data not shown). All UMNJ60_06UM strains reacted with mAb 2C7 by western blot (data not shown) and by flow cytometry (see Results).

Mass spectrometry

Fresh chocolate agar plates were inoculated with bacteria harvested from cultures grown overnight for 15 h and bacteria were grown for 6 h. LOS was extracted, de-O-acylated and analyzed by MS as described previously (148).

Antibodies

Anti-LOS mAbs 2-1-L8 (henceforth referred to as mAb L8) (149), 17-1-L1 (referred to as mAb L1) (150), 3F11 (151) and 2C7 (138) have been described previously. A schematic of the epitopes recognized by these mAbs is provided in Fig. 1. mAb 2C7 was purified from tissue culture supernatants over protein A/G (Pierce). Affinity-isolated goat anti-human factor H (FH) was prepared from anti-FH antiserum (Complement Technology, Inc., Tyler, TX) by passage over FH-sepharose as described previously (152). Alkaline phosphatase conjugated anti-mouse IgG and anti-mouse IgM, and FITC-conjugated anti-mouse IgG and anti-goat IgG were from Sigma. mAb 104 that binds to domains 1 and 2 of the α chain of human C4b-binding protein (C4BP) (153) was provided by Dr. Anna M. Blom

(Lund University, Malmö, Sweden). mAb 104 blocks C4BP function (153) and also blocks C4BP binding to gonococcal PorB (154) when pre-incubated with serum. However, mAb 104 does not displace C4BP already bound to the gonococcal surface and was used as the detection reagent for C4BP binding, as previously described (154). C3 deposited on gonococci was detected with FITC-conjugated anti-human C3c (AbD Serotec / Bio-Rad), which detects both C3b as well as iC3b, at a dilution of 1:100. In order to demonstrate that mAb 104 blocked C4BP binding to bacteria, complement was incubated with mAb 104 (9 µg of mAb 104 was added to 30 µl of complement) on ice for 10 min, added to bacteria. C4BP bound to bacteria was detected with anti-human C4BP mAb 67 (provided by Dr. Anna M. Blom) that recognizes domain 4 of the α chain of C4BP, followed by anti-mouse IgG A647 (Sigma) both at a dilution of 1:100.

SDS-PAGE and western blotting

Protease K-digested bacterial lysates were separated on 12% Bis-Tris gels (Invitrogen) with MES running buffer (Invitrogen) and LOS was visualized by Silver Stain (Bio-Rad). LOS was transferred to PVDF (Millipore) by western blotting; membranes were blocked with PBS/1% milk for 1 h at 37 °C and probed with tissue culture supernatants containing anti-LOS mAbs 2C7, 3F11, L1 and L8 (described above) for 15 h at 4 °C, as described previously (155). mAb-reactive LOS bands were visualized with anti-mouse IgG-alkaline phosphatase (for mAbs 2C7, L1 and L8) or anti-mouse IgM alkaline phosphatase (for mAb 3F11).

Hexosaminidase treatment

To ascertain whether a terminal hexosamine (in this instance, GalNAc) was present on the *lgtD*-‘ON’ (D+) mutants, bacteria were suspended in water, frozen at -20 °C and thawed at 37 °C to osmotically lyse them and treated with 10 U DNase I in DNase buffer (Ambion) for 60 min at 37 °C. Treatment with DNase I was carried out to reduce viscosity of the sample prior to electrophoresis. Proteins were digested with 1 mg/mL protease K (Calbiochem) in SDS (final concentration 0.01%) for 1 h at 50 °C. Protease K activity was destroyed by heating at 100 °C for 20 min. Terminal N-acetyl hexosamine from LOS was released by treating the sample with 30 U β -N-acetylhexosaminidase in G2 buffer (both from New England Biolabs) for 15 h at 37 °C. Samples were electrophoresed on a 16.5% Criterion™ Tricine gel (Bio-Rad) at 100 V at 4 °C and LOS was visualized with silver staining as described above.

Human complement

Blood was obtained from human volunteers (informed consent approved by the University of Massachusetts Institutional Review Board) and serum immunodepleted of IgG and IgM by passage over Protein A/G plus agarose (Pierce, USA) and anti-human IgM agarose columns (Sigma) to prepare complement (156). The flow through was spin concentrated, equilibrated with PBS/0.1 mM EDTA and sterilized by passage through a 0.22 μ m filter (Millipore, USA). Hemolytic activity was determined using the Total Haemolytic

Complement Kit (Binding Site, UK). Flow cytometry using FITC-conjugated anti-human IgG and anti-human IgM (Sigma) showed no detectable IgG or IgM binding in the depleted serum to strains that were used in experiments. Antibody depleted serum (henceforth referred to as “complement” or C') was aliquoted and stored at -80 °C until use. In some experiments C4BP function and binding to gonococci was blocked by adding mAb 104 (154, 157) to complement (30 µg of mAb 104/100 µL of complement).

Flow cytometry

Flow cytometry was used to measure binding of mAb 2C7, C4b-binding protein (C4BP) and deposition of complement C3 to bacteria as described previously (158-160). All Abs were diluted in Hanks Balanced Salt Solution containing 2 mM each of Ca^{2+} and Mg^{2+} (HBSS⁺⁺). Data were collected from a BD LSRII or FACSCalibur instrument (BD Biosciences, Franklin Lakes, NJ) and analyzed using a FlowJo analysis software program (version 7.2.5; Tree Star, Ashland, MA).

Serum bactericidal assays

Serum bactericidal assays were performed as described previously (144, 155). Briefly, bacteria harvested from an overnight culture on chocolate agar plates were re-passaged onto fresh chocolate agar and grown for 6 h at 37°C in an atmosphere of 5% CO₂. Approximately 2000 CFU gonococci in HBSS⁺⁺ were

incubated with complement (concentration specified for each experiment) either in the presence or absence of mAb 2C7 (concentration specified for each experiment). In some experiments, C4BP function was blocked by pre-incubating complement with 30 µg/mL of mAb 104 as described above. Final bactericidal reaction volumes were maintained at 150 µL. Aliquots of 10 µL were plated onto chocolate agar plates in duplicate at the beginning of the assay (t_0) and again after incubation at 37°C for 30 min (t_{30}). Survival was calculated as the number of viable colonies at t_{30} relative to t_0 .

Opsonophagocytosis assay using human PMNs

Human neutrophils were isolated from human blood over a Percoll gradient and opsonophagocytosis assays performed using freshly isolated IL-8 primed adherent neutrophils as previously described (161). Briefly, bacteria were incubated with mAb 2C7 (4 µg/mL) and/or human complement (20%), or with HBSS⁺⁺ alone (controls) for 15 min at 37 °C to permit IgG binding and C3 deposition. Reaction mixtures were added to IL-8 primed, adherent PMNs at an MOI of 1:1 and centrifuged at 400 *g* for 4 min at 10 °C to achieve synchronous infection (161). Cells were washed once with PBS/0.5% BSA, placed into RPMI with 10% heat-inactivated FBS and warmed to 37 °C. Cells were washed and lysed using 1% saponin in PBS at 0 min (taken immediately after the 10 °C centrifugation step) and parallel wells were similarly treated at 60 min, serially

diluted in GC broth and plated to determine viable CFU. Survival was expressed as the percent of CFU at 60 min relative to CFU at 0 min.

Statistical analysis

Comparisons between two groups were made using the two-tailed unpaired t test. One-way ANOVA was used to compare multiple groups; pairwise comparisons were made by Tukey's post-hoc test, while comparisons with a control group were made by Dunnett's test. Two-way ANOVA was employed to compare groups when time or concentrations were independent variables.

Results

Characterization of the LOS of the mutant strains

A schematic of potential gonococcal LOS structures, the relevant enzymes involved in biosynthesis of the outer core and the specificity of anti-LOS mAb(s) used to characterize LOS glycan extensions are shown in Fig. 1A. Phase variable expression of *lgtA*, *C* and *D* leads to variation in the HepI glycan extensions; HepI 2-Hex (*lgtA*, *C* and *D* all 'OFF'), HepI 3-Hex (*lgtA* 'OFF', *lgtC* 'ON' and *lgtD* 'ON' or 'OFF'; expression of *lgtD* is extraneous in an *lgtA* 'OFF' background), HepI 4-Hex (*lgtA* 'ON', *lgtC* and *D* 'OFF') and HepI 5-Hex (*lgtA* and *D* 'ON', *lgtC* 'OFF'). Phase variable expression of *lgtG* controls expression of lactose on HepII. To investigate the role of HepI glycan extensions on the function of mAb 2C7, we constructed a series of mutants in the background of MS11 4/3/1 in which the phase variable *lgt* loci (*lgtA*, *C*, *D* and *G*) were genetically fixed either 'ON' or 'OFF'. *Lgt* loci were fixed 'ON' by mutating the repetitive homopolymeric sequence found in each gene such that the homopolymer was removed but the coding sequence was not altered, as previously described (162). *Lgt* loci were fixed 'OFF' by deletion (*lgtA*, *lgtD*), insertional inactivation (*lgtG*) or by removing the homopolymeric sequence and inserting stop codons in all three reading frames (*lgtC*).

The LOS structures expressed by individual mutants were characterized by western blotting using the anti LOS mAbs described in Fig. 1A; relative masses

of the LOS(s) were determined by SDS-PAGE (Fig. 1B). For simplicity, we refer to the mutants used in this study by their longest predicted Hepl structures assuming activity of all expressed Lgt enzymes. The 'ON' and 'OFF' status of *lgtG* is indicated as G+ and G-, respectively. For example, in lane 1 (Fig. 1B), the mutant with *lgtA* and *lgtD* 'ON' is expected to have a 5-Hex Hepl structure (note the use 'Hex' in the text includes both hexoses and N-acetyl hexosamines). *lgtG* in this mutant is fixed 'ON', so the mutant is referred to as 5-Hex/G+. This simplified designation for each mutant is provided at the bottom of Fig. 1B and in Table 1.

Note that fixing an *lgt* 'ON' does not ensure that all of the LOS displayed on the bacterial surface will be substituted with the glycan added only by the encoded *lgt* enzyme(s) because transport of 'incomplete' LOS molecules to the outer membrane from the site of assembly on the cytoplasmic side of the inner membrane may occur prior to the addition of a glycan by all Lgt(s) that are fixed 'ON'. The amount and efficiency of each *lgt* enzyme will determine the ratio of 'complete' to 'incomplete' LOS expressed (163). An example of the transport of 'incomplete' LOS, shows that >50% of the LOS expressed by the two strains in which *lgtD* has been locked 'ON' (5-Hex/G+ and 5-Hex/G-) reacts with mAb 3F11 and represent 4-Hex structures with a terminal lactosamine (the lower, more prominent band in lanes 1 and 2 shown in the Silver stain row; Fig. 1B) indicating that, despite expression of *lgtD*, the majority of LOS in these mutants is exported to the surface prior to the addition of the terminal GalNAc to LOS. Another

example is provided by mAb L8, which reacts specifically with LOS structures that contain a lactose on HepI and no glycans from the 3-position of HepII (i.e., *lgtA* 'OFF' and *lgtG* 'OFF' respectively) (149). Thus, if all the LOS(s) expressed by mutants with *lgtA* 'OFF' and *lgtC* and/or *lgtG* 'ON' were substituted with a terminal $\alpha(1,4)$ -linked Gal on HepI and/or a proximal Glc on HepII, these mutants should not react with mAb L8. In fact, mAb L8 reacted with all three mutants that had *lgtA* 'OFF' and *lgtC* and/or *lgtG* 'ON' (lanes 5, 6 and 7 in the L8 blot in Fig. 1B), indicating export of LOS structures to the surface in these mutants prior to addition of: Glc on HepII by LgtG (lanes 5 and 7) and/or the distal $\alpha(1,4)$ -linked Gal on HepI (LgtC; lane 5). By contrast, fixing *lgtA* 'ON' (*lgtA*+) did not result in any detectable 'short' LOS structures (no L8 reactive bands seen in lanes 2 and 4 (Fig. 1B; mutants with *lgtA* 'ON' and *lgtG* 'OFF'), suggesting that LgtA efficiently added GlcNAc to the proximal lactose on HepI.

Mass spectrometric analysis confirmed LOS(s) of HepII glycan extensions in the *lgtG* 'OFF' mutants and the presence of HepII glycans in the *lgtG* 'ON' mutants (**Supplemental Table S3**). Mass spectrometry also confirmed that all mutants expressed the expected HepI glycan extensions shown in Table 1, as well as 'incomplete' structures as noted above. Further evidence that supported the presence of a terminal HexNAc residue in the 5-Hex/G+ and 5-Hex/G- mutants was provided by β -N-acetyl hexosaminidase treatment, which resulted in almost complete disappearance of the highest molecular mass band on silver staining of their LOS (Fig. 1C).

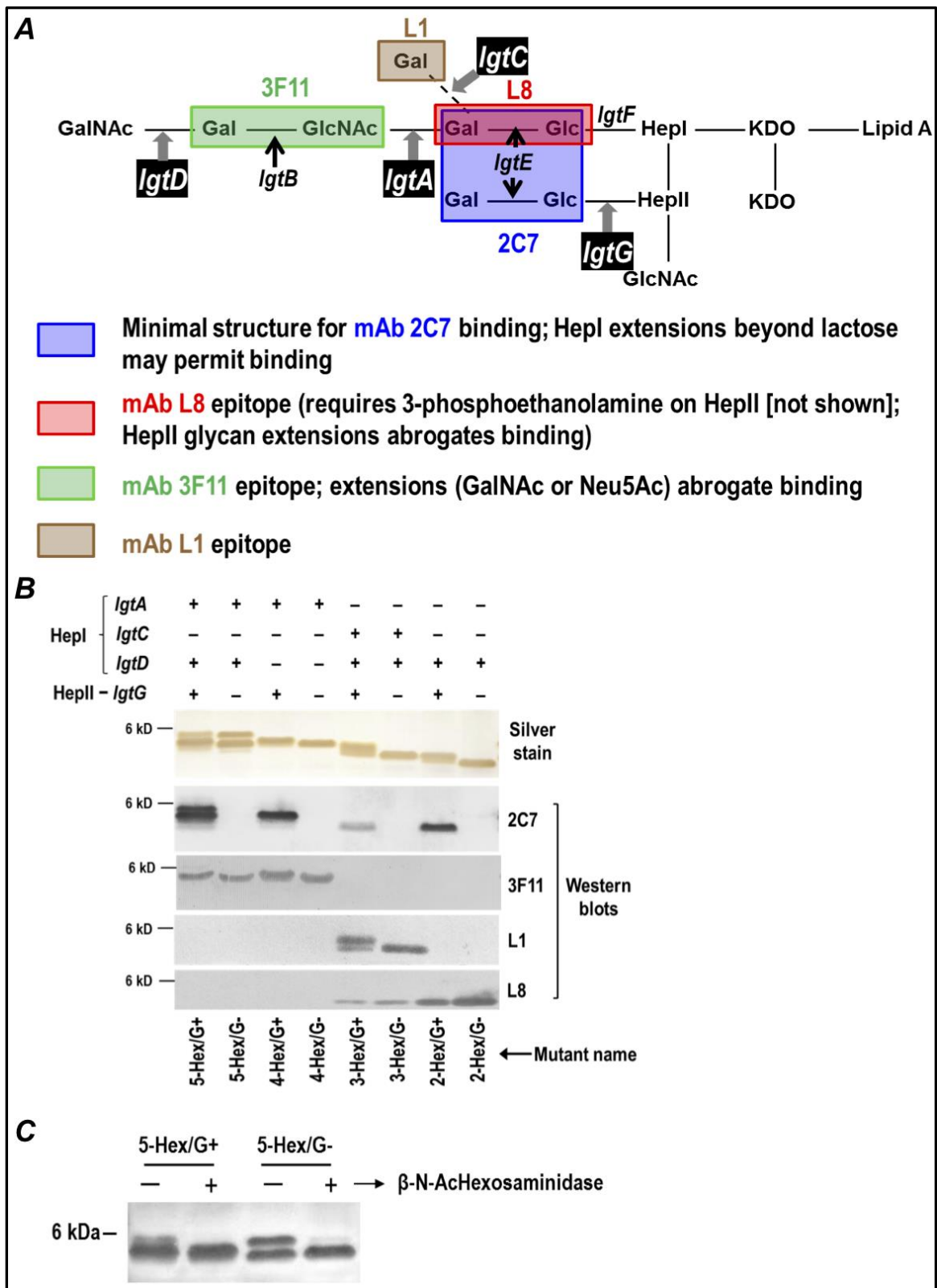


Figure 1. Characterization of the LOS of the MS11 mutants used in this study. **A.** Schematic representation of gonococcal LOS, relevant LOS glycosyltransferase (*lgt*) genes involved in its biosynthesis and the glycan structures recognized by anti-LOS mAbs. The four phase variable genes involved in glycan extensions *lgtA*, *C*, *D* and *G* are shown in black boxes. Specific structural requirements for binding of each of the four anti-LOS mAbs are indicated below the figure. **B.** Phenotypic characterization of the LOS mutants. Proteinase K-treated bacterial lysates were separated on 12% Bis-Tris gels and subsequently either stained with silver or transferred to PVDF by Western blotting and probed with specific anti-LOS mAbs. The genotypes of the mutants are indicated above the silver stain, while the simplified nomenclature for each of the mutants is indicated below the mAb L8 western blot. **C.** β -N-acetyl hexosaminidase treatment of 5-Hex mutants results in loss of the highest molecular mass LOS species. Bacterial lysates were treated with β -N-acetyl hexosaminidase (lanes marked “+”) or buffer alone (lanes marked “-”) and LOS was separated on 16.5% Criterion™ Tricine gel (Bio-Rad) and visualized by silver staining.

Table 1: Bacterial strains used in this study.			
Strain	Expected LOS phenotype	Description	Ref.
MS11 4/3/1	LOS structure not defined	MS11 with an IPTG inducible <i>pilE</i>	(142)
Derivatives of MS11 4/3/1			
5-Hex/G+	GalNAc-Gal-GlcNAc-Gal-Glc-HepI Gal-Glc-HepII	<i>lgtA</i> -ON ^A <i>lgtD</i> -ON ^B <i>lgtC</i> -OFF ^C <i>lgtG</i> -ON ^D	This study
5-Hex/G-	GalNAc-Gal-GlcNAc-Gal-Glc-HepI Unsubstituted HepII	<i>lgtA</i> -ON ^A <i>lgtD</i> -ON ^B <i>lgtC</i> -OFF ^C <i>lgtG::kan</i>	This study
4-Hex/G+	Gal-GlcNAc-Gal-Glc-HepI Gal-Glc-HepII	<i>lgtA</i> -ON ^A <i>lgtD</i> -del ^E <i>lgtC</i> -OFF ^C <i>lgtG</i> -ON ^D	This study
4-Hex/G-	Gal-GlcNAc-Gal-Glc-HepI Unsubstituted HepII	<i>lgtA</i> -ON ^A <i>lgtD</i> -del ^E <i>lgtC</i> -OFF ^C <i>lgtG::kan</i>	This study
3-Hex/G+	Gal-Gal-Glc-HepI Gal-Glc-HepII	<i>lgtA</i> -del ^F <i>lgtC</i> -ON ^G <i>lgtG</i> -ON ^D	This study
3-Hex/G-	Gal-Gal-Glc-HepI Unsubstituted HepII	<i>lgtA</i> -del ^F <i>lgtC</i> -ON ^G <i>lgtG::kan</i>	This study
2-Hex/G+	Gal-Glc-HepI Gal-Glc-HepII	<i>lgtA</i> -del ^F <i>lgtC</i> -OFF ^C <i>lgtG</i> -ON ^D	This study
2-Hex/G-	Gal-Glc-HepI Unsubstituted HepII	<i>lgtA</i> -del ^F <i>lgtC</i> -OFF ^C <i>lgtG::kan</i>	This study
2 → 3-Hex/G+	Gal-Gal-Glc-HepI; Gal-Glc-HepII	2-Hex/G+ with <i>lgtC</i> -ON ^G	This study
3 → 2-Hex/G+	Gal-Glc-HepI Gal-Glc-HepII	3-Hex/G+ with <i>lgtC</i> -OFF ^C	This study
UMNJ60_06UM	LOS structure not defined	Nanjing, PRC 2013; symptomatic male with urethritis. Intermediate resistance to ceftriaxone (E-test MIC = 0.38 µg/ml and disc = 35 mm (sensitive ≥ 35mm))	(143)
Derivatives of UMNJ60_06UM			
UMNJ60 2-Hex	Gal-Glc-HepI Gal-Glc-HepII	UMNJ60_06UM <i>lgtA::kan</i> ; expresses 2-Hex on HepI	This study
UMNJ60 3-Hex	Gal-Gal-Glc-HepI Gal-Glc-HepII	UMNJ60_06UM <i>lgtA::kan</i> ; expresses 3-Hex on HepI	This study

^A *lgtA*-ON; G₁₂ → GGGCGGAGGTGG

^B *lgtD*-ON; G₁₃ → GGGCGGAGGTG

^C *lgtC*-OFF; G₁₄ → GGTGAGGGGGGGGG

^D *lgtG*-ON; C₁₁ → CCCCTCCGCCA

^E *lgtD*-del; 744 base pair (64 – 808 of coding sequence) deletion from *lgtD*

^F *lgtA*-del; 417 base pair (50 – 467 of coding sequence) deletion from *lgtA*

^G *lgtC*-ON; G₁₄ → GGGGCGGAGG

HepI glycan substitutions modulate binding of mAb 2C7

Binding of mAb 2C7 (concentrations ranging from 0.1 to 10 µg/mL) to the LOS mutants was studied by flow cytometry (FCM). The amount of mAb 2C7 bound to bacteria measured by FCM varied across the mutants (Fig. 2). The 2-Hex/G+ mutant showed maximum binding and 3-Hex/G+ the least; 4- and 5-Hex/G+ mutants bound 'intermediate' amounts of 2C7. Binding of mAb 2C7 requires lactose extension from HepII. As expected, none of the *lgtG* deletion ("G-") mutants showed binding above conjugate control levels (data not shown).

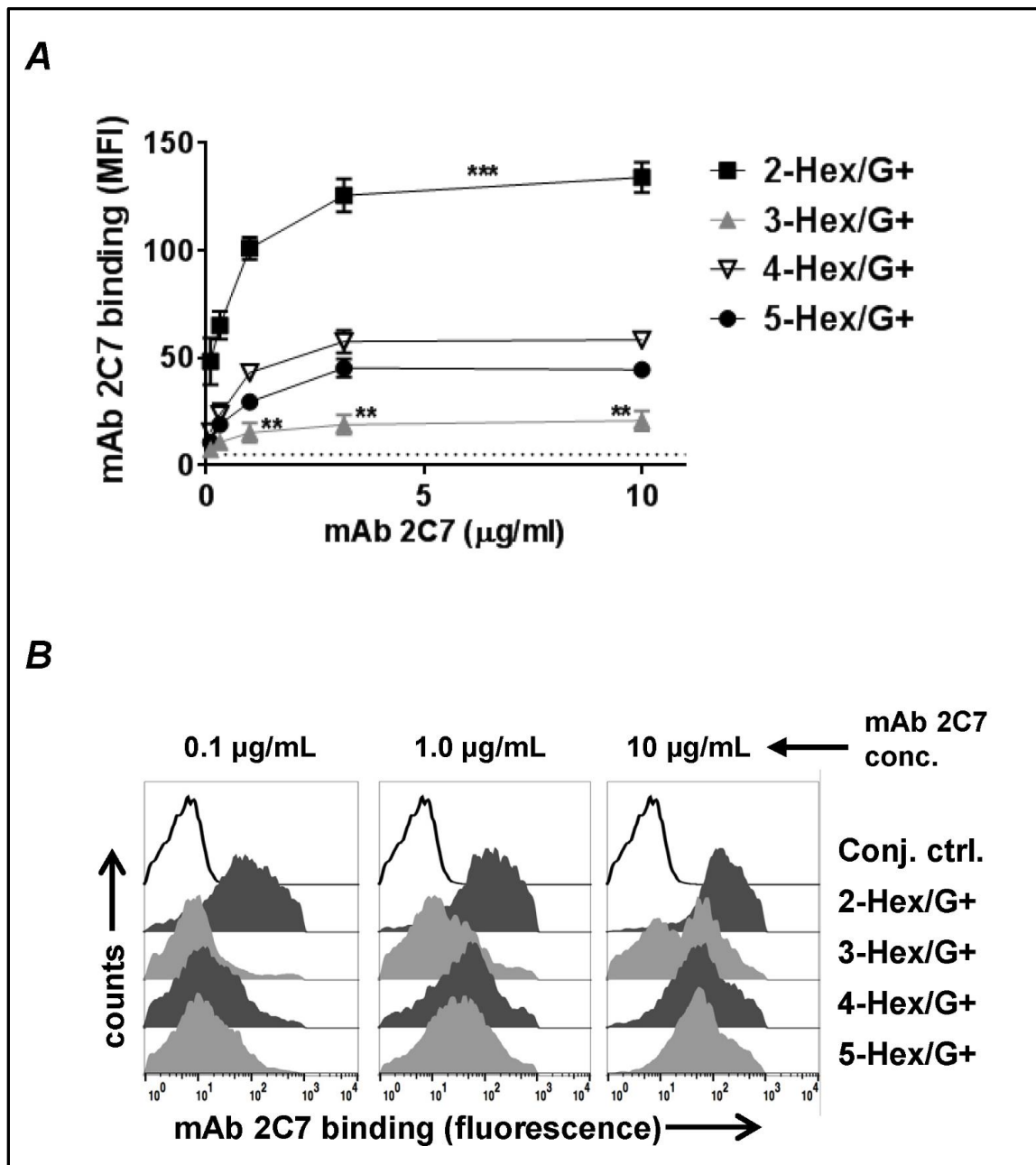


Figure 2. Hepl LOS glycan extensions modulate binding of mAb 2C7. The LOS mutants were incubated at 37 °C with increasing concentrations of mAb 2C7 for 30 minutes. Surface-bound mAb 2C7 was detected by flow cytometry (FCM) using FITC-conjugated anti-mouse IgG. A. Binding of mAb 2C7 (concentrations ranging from 0.1 to 10 μg/ml) to LOS mutants. Each data point represents the

mean of the median fluorescence intensities of 3 separate experiments (\pm SEM). Comparisons between the mutants at each dilution of mAb 2C7 were performed by two-way ANOVA with Tukey's post-test. ***, $P < 0.001$ for the 2-Hex/G+ versus all other mutants at each of the five concentrations tested. **, $P < 0.01$ for the 3-Hex/G+ mutant versus the 4- and 5-Hex/G+ mutants at the three concentrations indicated. Overall P values for interaction, row factor and column factors were all < 0.0001 . B. Representative histograms depicting mAb 2C7 binding at 0.1, 1.0 and 10 $\mu\text{g/mL}$. X-axis, fluorescence on a log₁₀ scale; Y-axis, counts.

Hepl glycan extensions modulate bactericidal efficacy of mAb 2C7

The ability of mAb 2C7 to kill each of the four G+ mutants was studied next. Bacteria were incubated with either 2 µg/mL or 4 µg/mL of mAb 2C7 and 20% human complement (normal human serum depleted of IgG and IgM); survival at 30 min was measured by bacterial CFU relative to CFU at 0 min (Fig. 3). As expected, control reactions (no mAb 2C7 added) showed no killing (>100% survival). Additional controls with mAb 2C7 alone (no added complement) or heat-inactivated complement also showed no killing (data not shown). The 2-Hex/G+ mutant showed >90% killing in the presence of 2 µg/mL of mAb 2C7; the 3-Hex/G+ mutant was fully resistant (>100% survival) to 4 µg/mL of mAb 2C7. The 4-Hex/G+ and 5-Hex/G+ mutants showed an intermediate pattern – i.e., resistance ($\geq 50\%$ survival) to 2 µg/mL of 2C7, but sensitivity (<50% survival) to 4 µg/mL of 2C7 (in this instance, >90% killing was observed). The bactericidal data followed a hierarchy similar to that seen with mAb 2C7 binding (Fig. 2).

Binding of the classical pathway inhibitor C4b-binding protein (C4BP) to gonococci modulates the efficacy of mAb 2C7 (155) and could have contributed to differences in susceptibility to mAb 2C7. We measured binding of C4BP to the four G+ mutants using heat-inactivated serum as a source of C4BP and found that all G+ mutants bound high and similar amounts of C4BP (Supplemental Fig. S1). These findings are consistent with prior data showing that MS11 and its LOS

derivatives that expressed at least 2 hexoses from Hepl bound C4BP well (154, 164).

The gonococcal genome contains over 100 phase variable genes. To confirm that differences in the binding of mAb 2C7 and killing between the 3-Hex/G+ and 2-Hex/G+ mutants were specifically related to LOS structure; *lgtC* was fixed 'ON' in the 2-Hex/G+ strain permitting addition of Gal- α (1,4) to Hepl (strain designated as 2 \rightarrow 3-Hex/G+) and *lgtC* was fixed 'OFF' in the 3-Hex/G+ strain, which blocked addition of Gal- α (1,4) to Hepl (strain designated as 3 \rightarrow 2-Hex/G+). The LOSs expressed by the mutants were verified by silver staining and with western blots using mAbs L8 and L1 (Fig. 4A). The two mutants, 2 \rightarrow 3-Hex/G+ and 3 \rightarrow 2-Hex/G+, were next examined for their ability to bind and be killed by mAb 2C7 (Fig. 4B and 4C). The results recapitulated those seen with the 3-Hex/G+ and 2-Hex/G+ mutants, respectively.

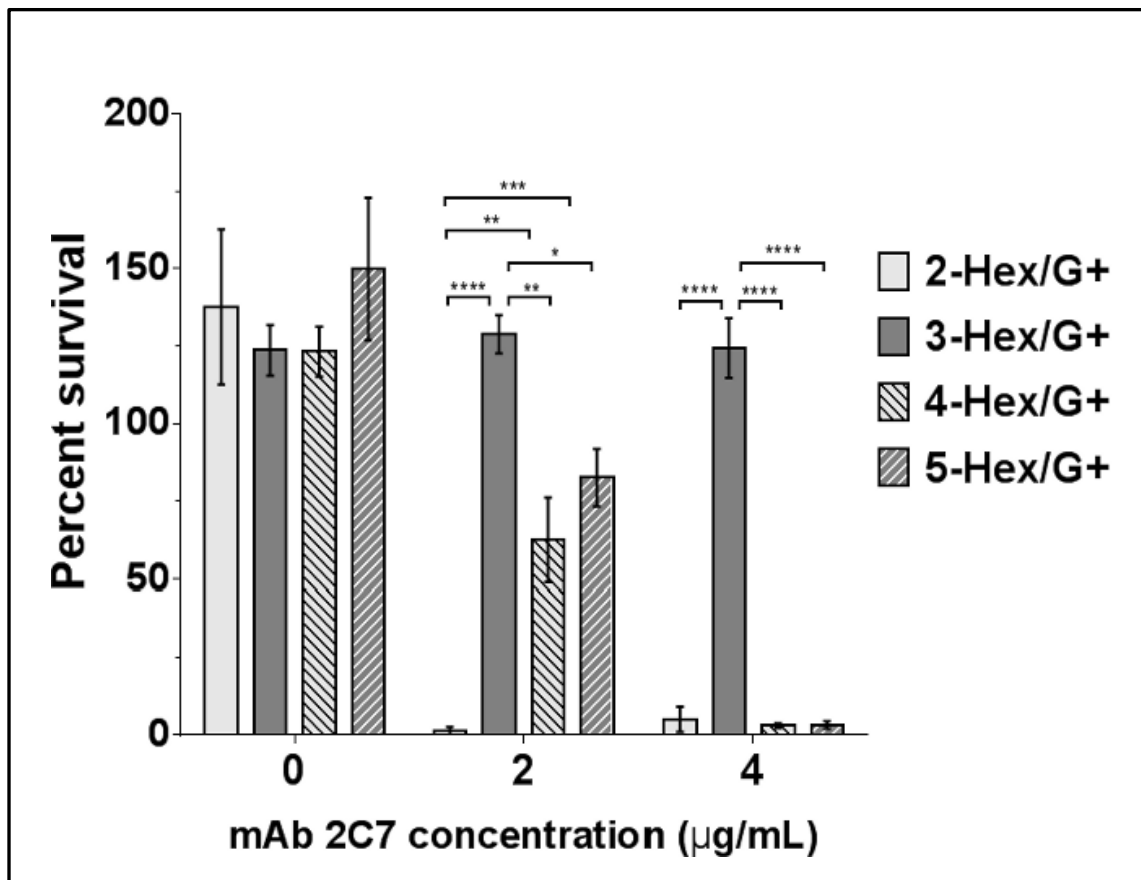
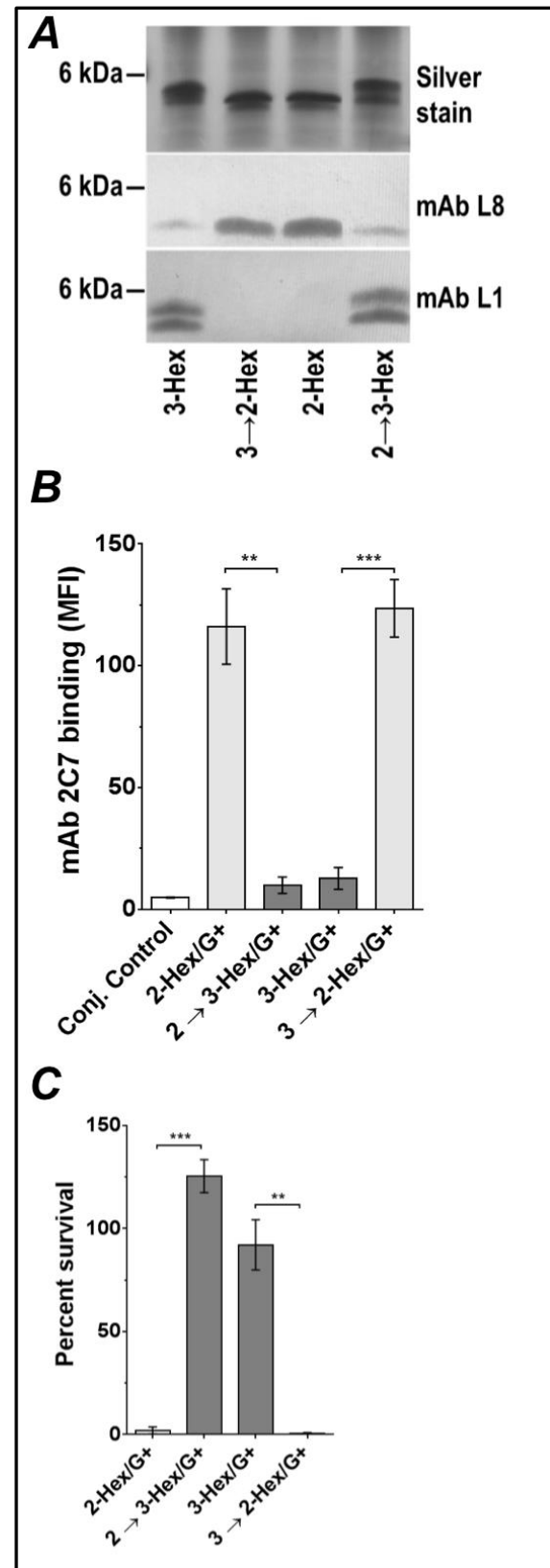


Figure 3. Hepl glycan extensions affect C'-dependent bactericidal activity by mAb 2C7. Each mutant was incubated with 20% (v/v) human complement (C') in HBSS⁺⁺ at 37 °C for 30 minutes either in the absence of or presence of 2 or 4 μg/mL mAb 2C7. Percent survival was calculated as the number of CFU at 30 min relative to the number of CFU at 0 min. Each bar represents the percent survival (mean of 3 independent experiments ±SEM). Comparisons among the mutants at each of the mAb 2C7 concentrations tested were made by two-way ANOVA and pairwise comparisons were made with Tukey's post-hoc analysis. Overall P values for interaction, row factor and column factors were all <0.0001. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001.

Figure 4. Confirmation of extreme mAb 2C7 binding phenotypes. Genetic conversion of MS11 2-Hex/G+ to 3-Hex/G+ (2→3-Hex/G+) and 3-Hex/G+ to 2-Hex/G+ (3→2-Hex/G+) confirms decreased binding mAb 2C7 and increased resistance of 3-Hex/G+ to mAb 2C7-mediated complement-dependent killing. **A.** Verification of LOS expression by the mutants by silver staining and western blotting with mAbs L1 and L8. **B.** mAb 2C7 (4 µg/ml) binding to the 2- and 3-Hex 'conversion' mutants. mAb 2C7 binding was performed by FCM as described in Fig. 2. Each bar represents the mean (±SEM) of three independent experiments. An unpaired two-tailed t-test was used to compare the mutant pairs. **C.** Susceptibility of the 2- and 3-Hex 'conversion' mutants. Serum bactericidal assays were performed as described in Fig. 3 in the presence of 4 µg/mL of mAb 2C7 and 20% human complement. Each bar represents the mean (±SEM) of three independent experiments. An unpaired two-tailed t-test was used to compare the mutant pairs. **, P<0.01; ***, P<0.001.

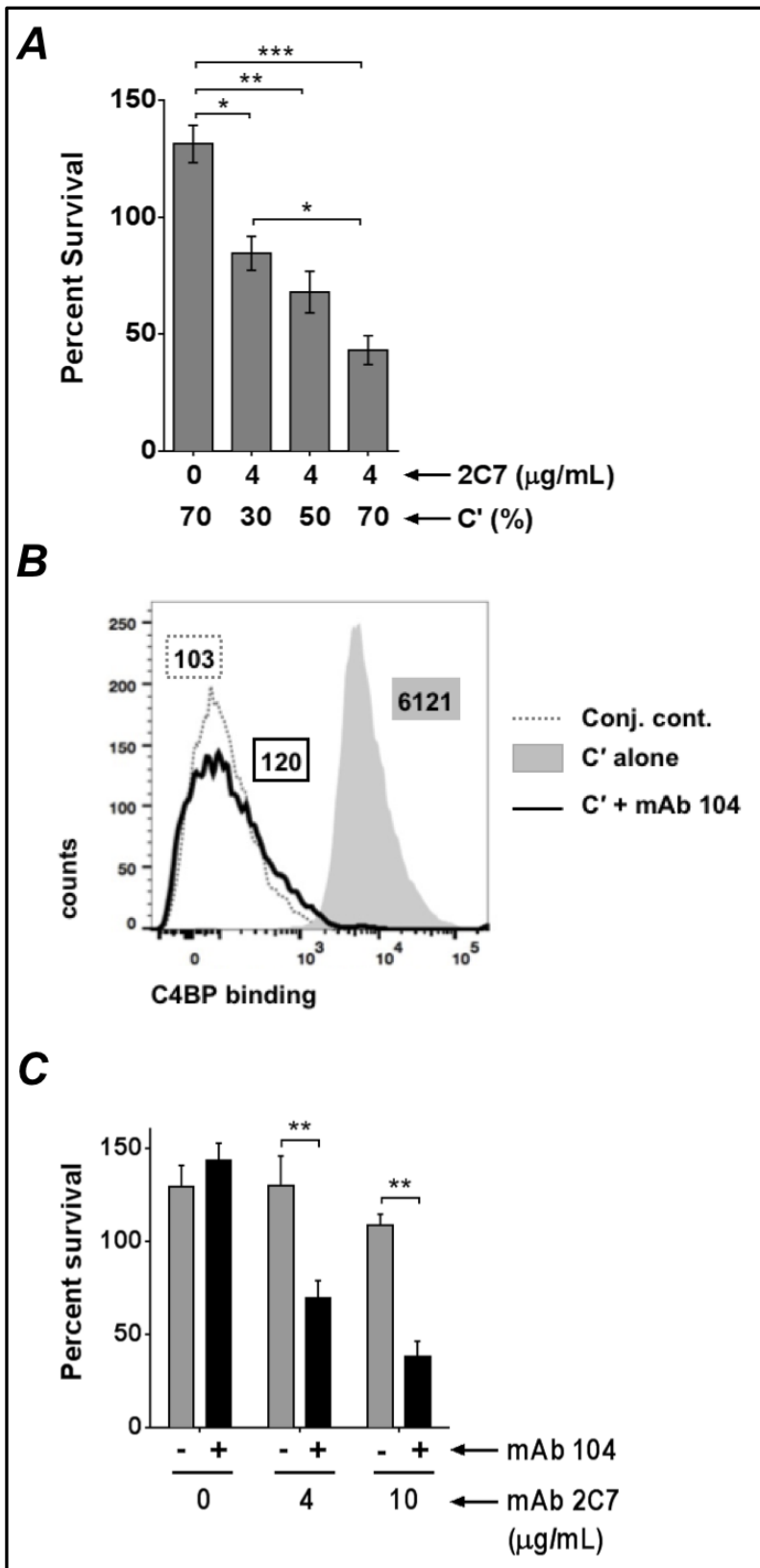


Serum resistance of 3-Hex/G+ is overcome by increasing complement concentrations or blocking C4BP binding

We next asked whether serum resistance of the 3-Hex/G+ mutant could be overcome by either increasing complement concentrations or by blocking C4BP binding to bacteria. As shown in Fig. 5A, in the presence of 4 µg/ml of 2C7, killing of 3-Hex/G+ was enhanced in a dose dependent manner by increasing the concentration of complement. Complement alone (mAb 2C7 absent), even at the highest concentration tested (70%), did not result in killing. Similar to our prior observations with strain MS11 (154), mAb 104 blocked C4BP binding to the 3-Hex/G+ mutant (Fig. 5B), which resulted in enhanced killing by mAb 2C7 compared to control reactions that lacked mAb 104 (Fig. 5C). Thus, increasing complement activation on the 3-Hex/G+ mutant either by increasing the concentration of complement or by decreasing complement inhibition by C4BP overcame its serum-resistant phenotype.

Figure 5. Serum resistance of MS11 3-Hex/G+ can be overcome by increasing complement concentrations or by inhibiting C4BP binding to bacteria. **A.**

Increasing complement concentrations enhances killing of the 3-Hex/G+ mutant in a dose-dependent manner. The 3-Hex/G+ mutant was incubated with 4 µg/ml of mAb 2C7 and increasing concentrations of human complement (C'; 30%, 50% and 70%) and bactericidal assays were performed as described in Fig. 2. The control reaction contained only the highest concentration of C' used (70%) without any added mAb 2C7. The Y-axis



shows percent survival. Each bar represents the mean (\pm SEM) of three independent experiments. Comparisons across different conditions of incubation were made by one-way ANOVA and pairwise comparisons were made with Tukey's post-test. The overall P value for the ANOVA was 0.0002. **B.** mAb 104 blocks C4BP binding to 3-Hex/G+. 3-Hex/G+ was incubated with either 20% C' alone or 20% C' plus mAb 104 (final concentration of 30 μ g/ml in the reaction mixture). C4BP bound to bacteria was detected with anti-C4BP mAb 67 followed by anti-mouse IgG A647. X-axis, fluorescence on a \log_{10} scale; Y-axis, counts. Numbers alongside histograms represents median fluorescence of the entire population and outlines or shading correspond to the histograms. **C.** Bactericidal efficacy of mAb 2C7 when C4BP binding to bacteria and function was blocked using mAb 104. C' was incubated with anti-C4BP mAb 104 to a final concentration of 30 μ g/ml at 4 °C for 15 min. The 3-Hex mutant was then incubated with (4 μ g/ml or 10 μ g/ml) or without mAb 2C7, followed by the addition of mAb 104-treated serum to a final concentration of 20%. Parallel control reactions included bacteria, mAb 2C7 and C' (no added mAb 104). The Y-axis denotes percent survival at 30 min. Comparisons between reactions that did or did not contain mAb 104 at each concentration of mAb 2C7 were made with a two-way ANOVA, with Sidak's multiple comparison test. Overall P values for interaction, row factor and column factor were 0.009, 0.0009 and 0.0014, respectively. Each bar represents the mean (\pm SEM) of three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

mAb 2C7 enhances C3 deposition and facilitates opsonophagocytosis of the 3-Hex/G+ mutant

C3 fragments, in particular iC3b deposited on bacteria enhance opsonophagocytosis. mAb 2C7 did not promote direct killing by complement of the 3-Hex/G+ mutant in serum bactericidal assays that used 20% complement (Fig. 3). However, we reasoned that mAb 2C7 mediated C3 deposition on the 3-Hex/G+ mutant supports opsonophagocytic killing and constitutes a potential mechanism of protection by vaccine Ab.

Total C3 (C3b and iC3b) deposition on the 3-Hex/G+ was measured by FCM; the three other G+ mutants were included as comparators. Bacteria were incubated with either 2 µg/ml or 4 µg/ml of mAb 2C7 and 20% complement; C3 deposited at 15 and 30 min was measured. In the absence of mAb 2C7 there was minimal C3 deposition on all mutants (median fluorescence <2-fold above baseline conjugate control levels [Fig. 6]). As expected, the 2-Hex/G+ mutant that was highly susceptible to complement-dependent killing showed the most rapid accumulation and the highest levels of C3 deposition. An intermediate amount of C3 was deposited on the 4-Hex mutant. The 3- and 5-Hex/G+ mutants bound the least; there was a trend toward less C3 on 3-Hex/G+ compared to 5-Hex/G+, however the differences were not significant.

The opacity (Opa) proteins of *N. gonorrhoeae* encompass a phase-variable family of proteins (gonococci possess 11 *opa* genes and can express three or

four Opa proteins simultaneously (165) that can engage CEACAM3 on PMNs and mediate opsonophagocytic killing independent of antibody and complement (166, 167). To address the potential role of mAb 2C7-dependent complement activation in facilitating opsonophagocytosis of the 3-Hex/G+ mutant, we recreated the 3-Hex/G+ LOS structure in the background of an Opa-negative MS11 strain (168). The 3-Hex/G+ Opa-negative MS11 mutant strain bound similar (low) levels of mAb 2C7 and was fully resistant (>100% survival) to killing by 4 µg/ml of mAb 2C7 plus 20% complement (Fig. 7; bar at far left), analogous to the 3-Hex/G+ in MS11 with its native *opa* genes intact. In the presence of both mAb 2C7 and complement (bar to the extreme right), PMNs caused a 60% decrease in bacterial survival ($P < 0.01$ compared to the control reaction with bacteria alone plus PMNs [second bar from left]). Compared to the control with bacteria and PMNs (second bar from left), reactions that contained bacteria, PMNs and 2C7 (third bar from the left) or bacteria, PMNs and complement (fourth bar from the left) did not show increased killing.

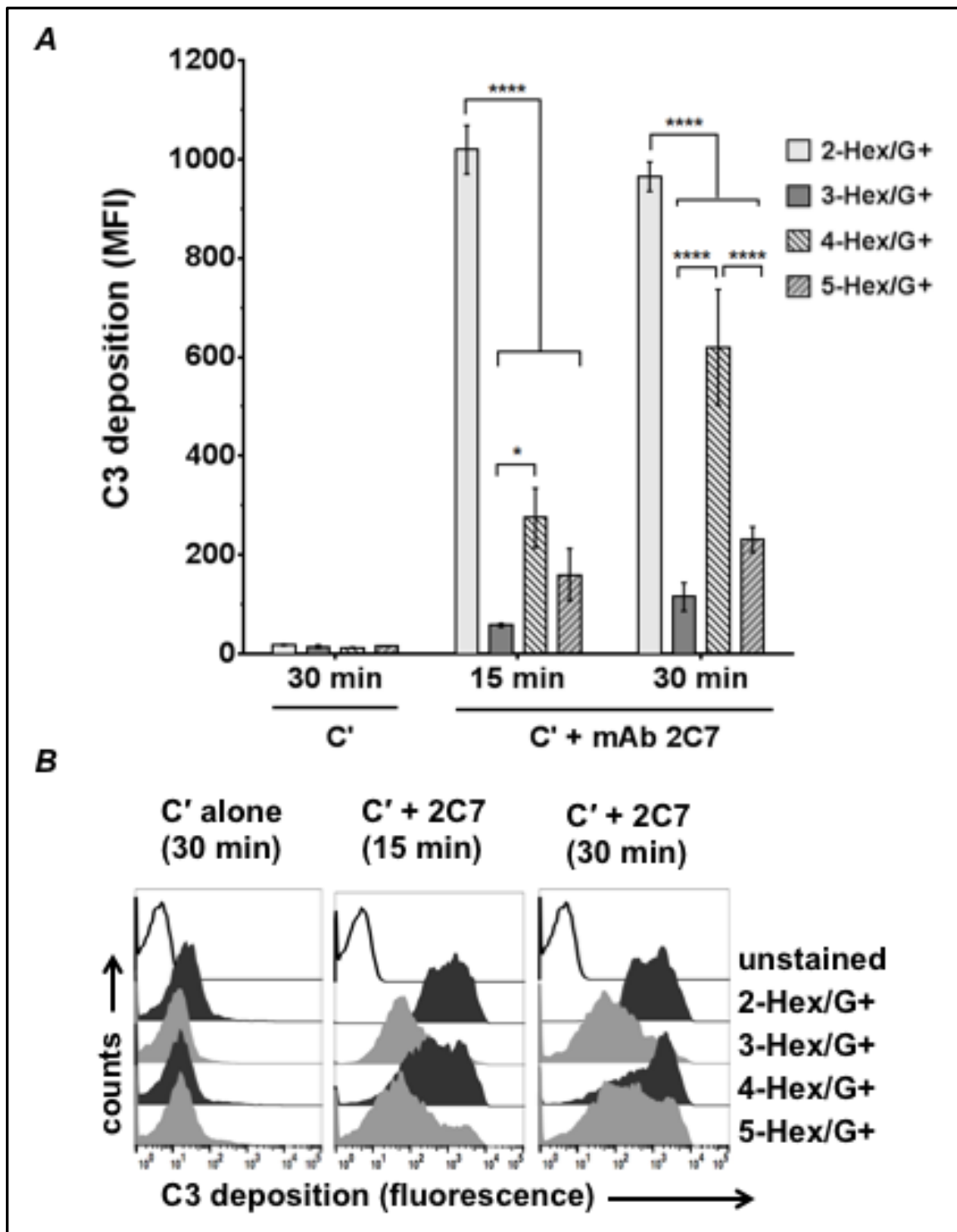
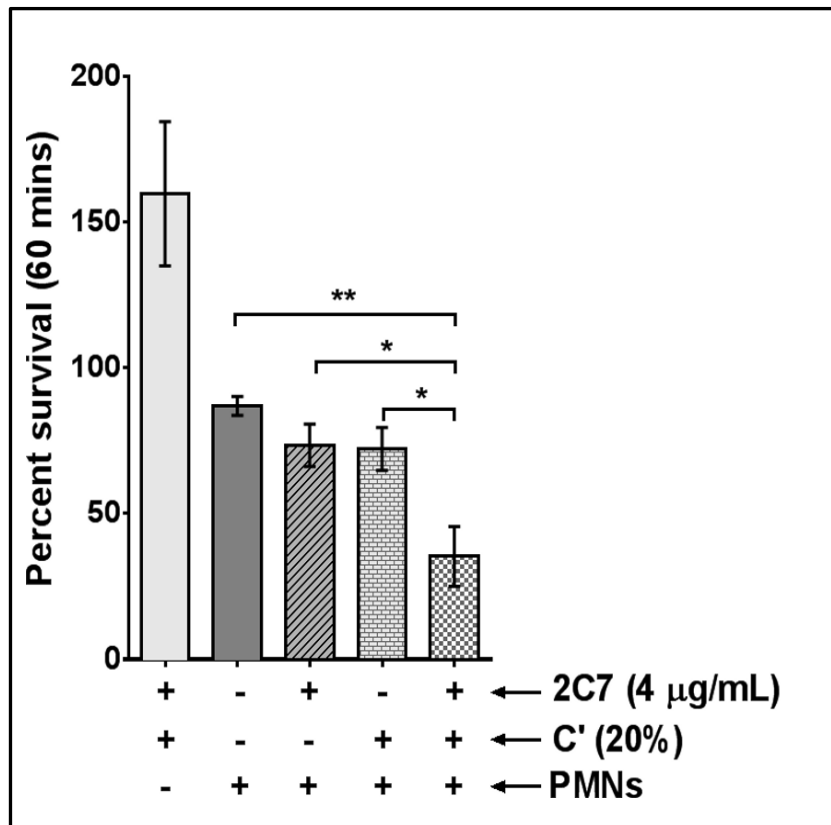


Figure 6. C3 deposition on the *IgtG*-ON' (G+) Hepl glycan LOS mutants. The four G+ LOS mutants with varying Hepl glycan extensions were incubated with

20% C' and 4 $\mu\text{g/ml}$ mAb 2C7 in HBSS⁺⁺ at 37 °C and total C3 (C3b and iC3b fragments) deposited on the bacterial surface at 15 or 30 min was measured by FCM with FITC-conjugated sheep anti-human C3c Ab (detects both C3b and iC3b). Control reactions included bacteria incubated with 20% C' alone (no mAb 2C7 present) for 15 and 30 min. The median fluorescence intensity was recorded. Similar C3 deposition was seen on bacteria incubated with C' alone for 15 min (not shown). Differences in C3 deposition across the mutants within each group was measured by two-way ANOVA and pairwise comparisons were made with Tukey's post-test. The data represent the mean ($\pm\text{SEM}$) from three independent experiments. P values for interaction, row factor and column factors were all <0.0001 . *, $P<0.05$; ****, $P<0.0001$. **B.** Representative histograms of a representative experiment in *A* is shown. X-axis, fluorescence on a \log_{10} scale; Y-axis, counts.

Figure 7. mAb 2C7 facilitates opsonophagocytosis of 3-Hex/G+ by PMNs. Freshly isolated IL-8 primed human PMNs adherent on plastic coverslips were synchronously infected with the 3-Hex/G+ mutant that had been pre-incubated with mAb 2C7 (4 μ g/ml) and/or C' for 15 min



37 °C at a MOI of 1. A reaction that contained bacteria, mAb 2C7 and C' (no PMNs) was also included (bar closest to the Y-axis). Percent survival (CFU at 60 min relative to CFU at 0 min) is shown on the Y-axis. Each bar represents the mean of the percent survival of 3 separate experiments (\pm SEM). A comparison of killing across the four groups that contained PMNs was performed by one-way ANOVA, with Dunnett's post-test used to make comparisons with the control reaction (bacteria plus PMNs; second bar from left). The overall P value for the ANOVA was 0.0064. *, $P < 0.05$; **, $P < 0.01$.

Relative resistance of a 3-Hex/G+ expressing lgtC phase-variant of a clinical isolate to killing by mAb 2C7

To ascertain if the decreased mAb 2C7 binding and increased resistance of the 3-Hex/G+ mutant was generalizable and not unique to strain MS11 alone, we identified 2-Hex and 3-Hex phase variants of an *lgtA* mutant ($\Delta lgtA$) of a minimally passaged clinical isolate called UMNJ60_O6UM. Two natural variants of UMNJ60_O6UM $\Delta lgtA$ were selected – one that expressed lactose on HepI (UMNJ60_O6UM 2-Hex; analogous with *lgtC* phase varied ‘OFF’ and therefore did not react with mAb L1) and one that expressed 3-Hex’ P^k-like LOS on HepI (UMNJ60_O6UM 3-Hex; analogous with *lgtC* phase varied ‘ON’ and therefore reacted with mAb L1; (169). Both variants had *lgtG* phase- ‘ON’ and therefore expressed lactose from HepII. The two variants were examined for mAb 2C7 binding and killing in a complement-dependent bactericidal assay.

UMNJ60_O6UM 2-Hex variant bound more mAb 2C7 than the UMNJ60_O6UM 3-Hex variant (Fig. 8A). UMNJ60_O6UM 3-Hex was also more resistant to complement-dependent killing by mAb 2C7 (Fig. 8B). The 2-Hex variant was killed >99% and 100% by complement in the presence of 2 µg/ml and 4 µg/ml of 2C7 respectively; the 3-Hex variant survived ~70% and ~50% under similar conditions.

Unlike the MS11 3-Hex/G+ mutant that was fully resistant (>100% survival) to killing by even 4 µg/ml of mAb 2C7 plus 20% complement (Fig. 3), the

UMNJ60_O6UM 3-Hex variant was partly susceptible to killing under similar conditions. As noted above, C4BP binds to certain *N. gonorrhoeae* strains, including MS11, and promotes serum resistance (Ref. (154) and Supplemental Fig. S1). To determine if UMNJ60_O6UM bound C4BP, we compared C4BP binding to the two UMNJ60_O6UM LOS variants with the corresponding MS11 LOS mutants. Both UMNJ60_O6UM variants bound significantly lower amounts of C4BP than the corresponding MS11 LOS mutants (Fig. 8C), thus providing a probable explanation for the greater sensitivity to complement of UMNJ60_O6UM variant 3-Hex compared to MS11 3-Hex/G+. Collectively, these data suggest that the 3-Hex Hepl structure negatively affects mAb 2C7 binding and function.

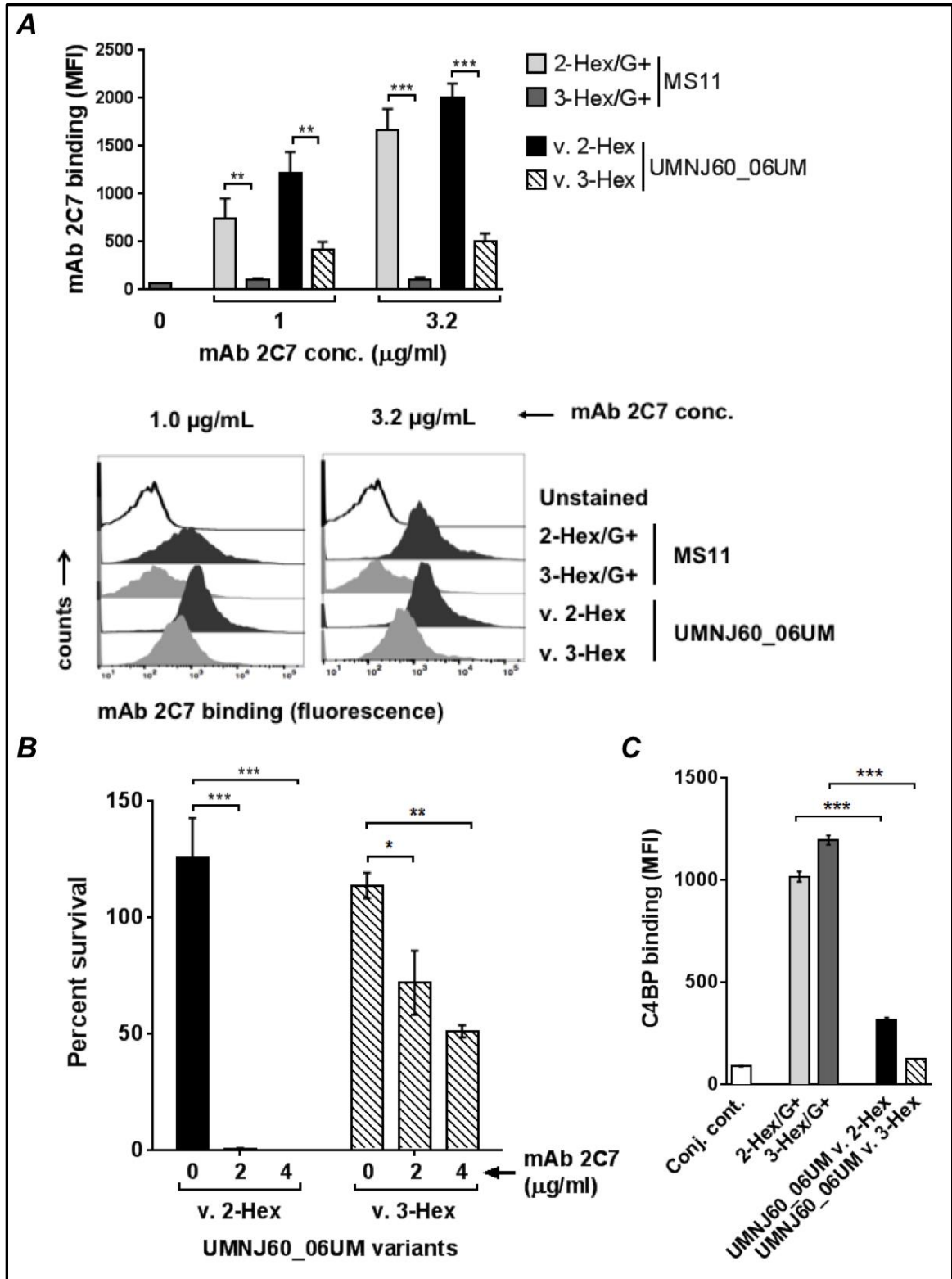


Figure 8. Validation of the MS11 3-Hex/G+ phenotype with a 3-Hex/G+ phase variant of UMNJ60_06UM, a recent clinical isolate of *N. gonorrhoeae*. Phase variants of an *lgtA* mutant of UMNJ60_06UM that expressed 3-Hex Hepl (UMNJ60 v. 2-Hex; *lgtC* 'ON', reacts with mAb L1, but not mAb L8) and that expressed 2-Hex Hepl (UMNJ60 v. 3-Hex; *lgtC* 'OFF', reacted with mAb L8 but not mAb L1) were selected. Both strains reacted with mAb 2C7 (consistent with *lgtG* 'ON'). **A.** The 3-Hex expressing variant of UMNJ60_06UM binds less 2C7 than the 2-Hex expressing variant. Bacteria were incubated with mAb 2C7 (1 $\mu\text{g/ml}$ or 3.2 $\mu\text{g/ml}$) and bound mAb was detected by FCM as described in Figure 2. The MS11 2-Hex/G+ and 3-Hex/G+ mutants were included as comparators. Each bar represents the mean of the median fluorescence intensities of 3 independent experiments ($\pm\text{SEM}$). Comparisons between the Hepl 2-Hex and Hepl 3-Hex-expressing isolates were made by the unpaired two-tailed t-test. Histograms from a representative experiment are shown on the right of the bar graphs. **B.** A 3-Hex variant of UMNJ60_06UM is more resistant to killing by mAb 2C7 than a 2-Hex variant. UMNJ60 v. 2-Hex and v. 3-Hex were incubated with increasing concentrations of mAb 2C7 (either 0, 2 or 4 $\mu\text{g/ml}$) plus 20% human complement and survival of bacteria at 30 min relative to CFU at time 0 min, was measured in serum bactericidal assays. Y-axis, percent survival. One-way ANOVA was used to compare killing of each of the variants at the different concentrations of mAb 2C7 tested; the v. 2-Hex and the v. 3-Hex groups were compared separately. The overall P values for the v. 2-Hex and v. 3-Hex groups were 0.0002 and 0.006, respectively. **C.** The Hepl LOS phase-variants of UMNJ60_06UM are weak C4BP binders. UMNJ60 v. 2-Hex and UMNJ60 v. 3-Hex were incubated with heat-inactivated NHS and C4BP bound to bacteria was detected by FCM. MS11 2-Hex/G+ and 3-Hex/G+ were included as comparators. The conjugate control ("Conj. cont.") represents a reaction mixture that lacked serum. Each bar represents the mean of 3 independent experiments $\pm\text{SEM}$. A two-tailed unpaired t-test was used to compare C4BP binding to the 2-Hex and 3-

Hex expressing strain pairs. Histograms from a representative experiment are shown to the right of the bar graph. The X-axis shows fluorescence on a \log_{10} scale and the Y-axis the events. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, ****, $P < 0.0001$.

Discussion

The role of LOS glycan extensions in the pathogenesis of *N. gonorrhoeae*, including their role in immune evasion, is well recognized. Much attention has been directed to LOS that expresses the lacto-*N*-neotetraose (LNnT) structure on Hepl, which mimics host paraglobosides (170). Unsialylated LNnT interacts with the asialoglycoprotein receptor and facilitates adhesion of gonococci to male urethral epithelial cells (32). A lectin-like interaction between the terminal lactosamine residue of LNnT and gonococcal opacity proteins (Opa) plays a role in inter-gonococcal adhesion and the degree of colony opacity (171). Gonococci possess a surface-exposed LOS sialyltransferase (172) that catalyzes the transfer of *N*-acetylneuraminic acid (Neu5Ac) from the donor molecule CMP-Neu5Ac present in host secretions (173, 174), on to the 3-position of the terminal Gal residue of LNnT. LNnT sialylation is involved in the inhibition of all three pathways of complement and enables gonococci to resist killing by natural IgG/IgM and complement in normal human serum (NHS), called serum resistance (83, 175, 176). Sialylation of gonococcal LNnT *in vivo* has been demonstrated by electron micrographs of organisms in human secretions (177). Schneider and colleagues also demonstrated the importance of phase variation of gonococcal LOS and LNnT sialylation in humans. They inoculated male volunteers intra-urethrally with a variant of strain MS11 that expressed a (non-sialylated) 2-Hex Hepl structure predominantly. At the onset of symptoms, several days later, almost all men shed bacteria that expressed LOS with

predominantly longer (including the 4-Hex HepI), sialylatable HepI structures (178). Recently, McLaughlin and colleagues found that gonococci present in urethral exudates of infected men displayed an *lgt* genotype that predicted sialylation of terminal lactosamine; *lgtA* was in-frame, while *lgtC* and *lgtD* were out-of-frame in most cases (179). The importance of LOS sialylation in pathogenesis has also been demonstrated in the mouse vaginal colonization/infection model; gonococcal mutants that lack LOS sialyltransferase (Lst) are outcompeted by their wild-type counterparts (158, 180). The efficacy of mAb 2C7 against phase variant and sialylated bacteria has been demonstrated both *in vitro* (organisms grown in media containing 2 µg/ml of CMP-Neu5Ac; (155)) and *in vivo* in mice, where LOS sialylation occurs (181)).

Diversity of surface antigens generated by phase variation confers a survival advantage to microbes and enables them to adapt to different niches in the host. Phase-variation of LOS also modulates resistance of *N. gonorrhoeae* to killing by NHS, independent of LOS sialylation (182-184). Expression of GalNAc distal to LNT (i.e., 5-Hex HepI; *lgtA* and *lgtD* 'ON') permits binding of natural IgM present in NHS (185) and enhances bacterial killing by complement (182). Although the terminal GalNAc enhances killing by NHS, gonococci that possess LOS with this terminal residue interact with macrophage galactose-type lectin (MGL) on dendritic cells. This may result in more pronounced Th2 and Th17 responses (186), instead of protective Th1 responses (67, 187). Expression of a truncated 3.6 kDa LOS (the 2-Hex [lactosyl] HepI), which also is a host glycan mimic, is

also associated with increased resistance to NHS because most humans lack natural (IgG/IgM) Ab against this epitope (183).

Our studies used mAb 2C7 and NHS depleted of IgG/IgM in bactericidal assays. In contrast to previous studies where mutants with short HepI glycan extensions (e.g., *lgtA* 'OFF') were more resistant to killing by NHS (182, 184), the 2-Hex/G+ mutant studied herein was more susceptible to killing by mAb 2C7 and complement (i.e., NHS depleted of IgG/IgM) than the three remaining mutants because it bound the most mAb 2C7, which resulted in overwhelming complement activation. By comparison, the 5-Hex/G+ strain, ordinarily more sensitive to killing by IgM in NHS (185), was relatively resistant to killing by mAb 2C7 plus complement because it bound less mAb 2C7. We replicated previous studies and showed killing (100% killing in 10% serum [IgG and IgM intact]) of the 5-Hex/G+ mutant; the 2- and 3-Hex/G+ strains were fully serum resistant (>100% survival in 10% NHS).

The 2C7 LOS epitope represents a promising vaccine candidate. Separately, we have shown that HepII glycan extension, required to generate the epitope, is also important in the mouse model of gonococcal colonization/infection, where an *lgtG* deletion mutant of strain FA1090 was shown to be less 'fit' (70). A role for HepII glycans in gonococcal pathogenesis is supported by the observation that >95% (96 of 101) of minimally passaged clinical isolates reacted with mAb 2C7 (138). A contemporary analysis of over 70 gonococcal isolates recovered from men with

urethritis attending an STD clinic in Nanjing, China, has substantiated these findings; all the recovered isolates bind mAb 2C7 (our unpublished observations).

A potential reason for (2C7) vaccine resistance would be selection of LOS variants that show decreased binding of Ab. One explanation would be natural selection of variant(s) expressing LOS with *lgtG* 'OFF'. However, loss of LgtG expression may reduce fitness and therefore not be favored (70). The hypothesis addressed in our study was that other different Hepl LOS structures may affect binding and function of mAb 2C7, an important consideration in predicting vaccine efficacy and coverage. Strains that expressed the P^k -like LOS structure (represented by the 3-Hex/G+ mutant) bound the least amount of mAb 2C7 and were relatively resistant to complement-mediated killing by mAb 2C7. With the exception of *lgtC*, which adds the terminal Gal on Hepl of the 3-Hex/G+ mutant via an α -linkage, glycans added by enzymes encoded by the *lgtABCDE* operon are β -linked (182, 188-190). The α -linked terminal Gal on Hepl of the 3-Hex/G+ may hinder access of mAb 2C7 to its epitope.

Several microbes bind C4BP, an inhibitor of the classical pathway, to evade killing by complement (191). MS11 binds C4BP and mAb 2C7 must surmount this barrier to mediate killing through the membrane attack complex insertion. The inhibiting role of C4BP was evident when C4BP binding to bacteria and C4BP function were blocked using mAb 104 resulting in increased killing of bacteria. Moreover, the 3-Hex variant of a clinical strain that bound low levels of

C4BP (UMNJ60_06UM) was more susceptible to killing by mAb 2C7 than the MS11 3-Hex/G+ that binds high levels of C4BP.

Despite the absence of killing through membrane attack complex, mAb 2C7 deposited sufficient C3 on 3-Hex/G+ bacteria to enable human PMNs to decrease CFU by >50%. Of note, maximal opsonophagocytic killing by PMNs required both Ab and complement; either one alone did not result in killing above baseline levels when bacteria only were incubated with PMNs. The *Neisserial* P^k-like LOS structure can also be sialylated (169, 192). Unlike sialylation of LNnT LOS, sialylation of P^k-like LOS does not enhance FH binding to bacteria and confers resistance to only low, but not high, concentrations of NHS (169). While McLaughlin and colleagues found that *lgtC* was out-of-frame in all 7 samples of *N. gonorrhoeae* obtained directly from male urethras (179), Mandrell reported that as many as 36 of 70 (51%) of strains surveyed *in vitro* bound mAb 3D9, which reacts with the P^k antigen (193). The advantage conferred by the gonococcal P^k-like LOS *in vivo* remains to be elucidated.

How mAb 2C7 decreases gonococcal burden and duration of infection *in vivo* – i.e., the specific contributions of direct complement-mediated killing, opsonophagocytosis or other novel mechanism(s) of Ab-mediated clearance – remain to be elucidated. Notwithstanding the differences in direct killing of the mutants by mAb 2C7 and complement, enhanced C3 deposition occurred on all the Hepl/G+ mutants; in particular, the 3-Hex/G+ mutant, which was not killed

directly, was opsonophagocytosed and killed. Our findings support the inclusion of the 2C7 LOS epitope in a vaccine candidate against *N. gonorrhoeae*.

Acknowledgements

We thank Ashild Vik and Michael Koomey (University of Oslo) for providing strain MS11 4/3/1, Daniel C. Stein (University of Maryland) for the Opa-negative MS11 mutant and F62 Δ *lgtA* *lgtG*⁺, William M. Shafer (Emory University) for providing FA19 *lgtG::kan*, Anna M. Blom (Lund University) for mAbs 67 and 104, Alison K. Criss (University of Virginia) for advice with opsonophagocytosis assays. We thank Sunita Gulati for mAb 2C7, and Nancy Nowak and Bo Zheng (all from the University of Massachusetts, Worcester) for expert technical assistance.

Supplementary Materials

Supplemental Table S1: Primers used in this study.

Primers	5'-3' Sequence
IgtG_F	CTATCTGTACGACGTTTGA AAAATTGC
IgtG_R	CCCCGTATTTAAAGGATAAAGGCAAAA
IgtG530	CGCATTACCTACCCCTCACGCAC
IgtG1729	TCGTACGACGTTTGA AAAATTGC
Lal-6	GCGACAGGACGGGTTGTAGTTCAG
Lal-7	GCGACGGACGCTACACATTGGAT
IgtA Fwd-Ext	AGGCAATTTCCAACTGCTTTGTCCGA
IgtA Rev-Ext	GTTTGGCGGTATTCAGGCTGTGGAAC
IgtC Fwd-Ext	TATTTGAGCGGAGTGAAAAAGCCTGC
IgtC Rev-Ext	ATCCAAGTTGGCCAAAGTCTGATTCAC
IgtD Fwd-Ext	TCAAAATGTCCTGCGAATGGGTGGAAC
IgtD Rev-Ext	AGCCAAGCTGATAACGTGGTTTGCAT
IgtA-On Fwd ^A	ATTGGCAAAGTCGGG <u>CGG</u> AGGTGGATATATTGCGCGC
IgtA-On Rev ^A	GCGCGCAATATATCC <u>ACCTCCG</u> CCCCGACTTTGCCAAT
IgtC-On Fwd ^A	CGCCAATTTGCGGGG <u>CGGAG</u> GAATATCCGCTTT
IgtC-On Rev ^A	AAAGCGGATATT <u>ACCTCCG</u> CCCCGCAAAATGGCG
IgtC-Off Fwd ^A	CCGCCAATTTGCGGTGAGGGGGGGTAATA
IgtC-Off Rev ^A	TATTACCCCCCTC <u>ACCG</u> CAAATGGCGG
IgtD-On Fwd ^A	GAATTGGCAAAGTCGGG <u>CGG</u> AGGTGAATATATTGCGCGAC
IgtD-On Rev ^A	GTGCGCGCAATATAT <u>ACCTCCG</u> CCCCGACTTTGCCAATC
IgtA Fwd-Int ^B	<i>AAAAGCGGGACAGCCGTATCAAAA</i> CCCGGGATTGAGAAAATCGTGGGCGAGATGG
IgtA Rev-Int ^B	<i>CCAATCTCGCCACGATTTCTCAA</i> CCCGGGATTTGATACGGCTGTCCCGCTTTT
IgtC Fwd-Int ^B	<i>GAGATGGACATCGTATTTGCGGCAGAC</i> CCCGGGCCTTATGGGATACCGATTGGGCGGTA
IgtC Rev-Int ^B	<i>TACCGCCCAAACTGGTATCCCATAGG</i> CCCGGGGTCTGCCGCAAAATACGATGTCCATCTC
IgtD Fwd-Int ^B	<i>GTGAATCAGACTTGGCGCAACTTGGAT</i> CCCGGGCATGAAGACATTGTCCCGTTTCCCT
IgtD Rev-Int ^B	<i>AGGGAAAACGGCGACAATGTCTTCATG</i> CCCGGGATCCAAGTTGCGCCAAGTCTGATTCAC

^AIndicates primers for mutagenesis; Single base substitutions are **underlined in bold**, consecutive bases *underlined in italics* span short deletions that render the sequence in frame for full length protein translation.

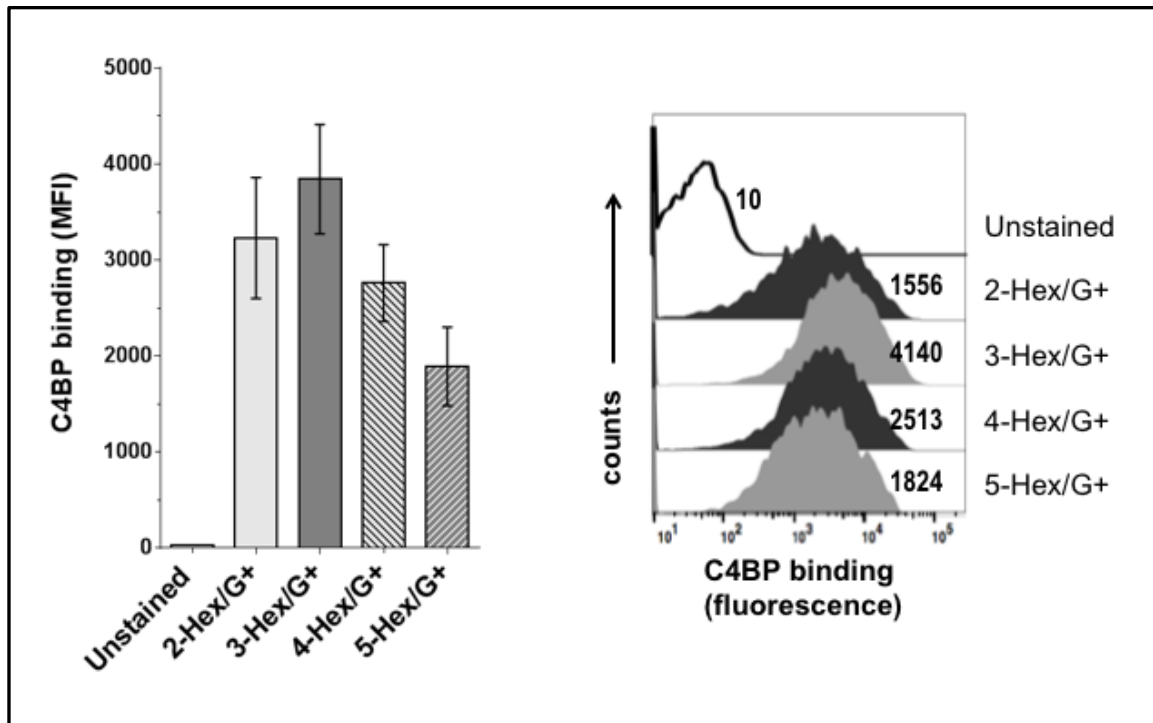
^BSmaI site – **CCCGGG**; Primer regions corresponding to the indicated *Igt* are indicated in **bold**, overlap regions are indicated in *italics*.

Supplemental Table 2: Plasmids used in this study.	
Plasmids	Relevant characteristics and construction details
pFLOB4270 (146)	Two-step mutagenesis system used to construct unmarked deletions or mutations. Contains an <i>Erm^R-Sm^S</i> cassette that encodes resistance to <i>Erm</i> and sensitivity to <i>Sm</i> (<i>rpsL_{F62}</i>). Used to construct unmarked mutations in <i>lgtG</i> , <i>C</i> , <i>A</i> and <i>D</i> .
lgtG constructs	
pRYGW2	Contains wildtype <i>lgtG</i> from <i>Ng</i> strain 398079 amplified with primers lgtG530 and lgtG1729 in pCR2.1-TOPO (Invitrogen, Carlsbad, CA). Analogous to pRYGW1, described previously (70). Used to create pRYGW2ES1.
pRYGW2ES1	pRYGW2 with the <i>Erm^R-Sm^S</i> cassette that encodes resistance to <i>Erm</i> (<i>ErmC</i>) and sensitivity to <i>Sm</i> (<i>rpsL_{F62}</i>) from pFLOB4300 ² . The <i>Erm^R-Sm^S</i> cassette was liberated with PvuII and cloned into the <i>Stu</i> I site of pRYGW2. Used to construct MS11 4/3/1 <i>lgtG</i> locked ON.
plgtG+	Contains <i>lgtG</i> locked ON (<i>C</i> ₁₁ → CCCCTCCGCCA); <i>lgtG</i> + was amplified from <i>Ng</i> F62 Δ <i>lgtA lgtG</i> + (194) with primers lal6 and lal7 and cloned into in to pCR2.1 TOPO (Invitrogen). Used to create <i>lgtG</i> locked ON.
lgtA constructs	
plgtA	Contains wildtype MS11 4/3/1 <i>lgtA</i> amplified with lgtA Fwd-Ext and lgtA Rev-Ext and cloned into pCR2.1 TOPO (Life Technologies, USA). Used as a template to construct <i>lgtA</i> locked on by site directed mutagenesis with primers lgtA-ON FWD and lgtA-ON REV.
plgtA-ES	Contains <i>lgtA</i> with <i>Erm^R-Sm^S</i> from pFLOB4300 inserted into an engineered <i>Sma</i> I site in the homopolymeric tract. Used to construct unmarked <i>lgtA</i> mutations (ON and OFF).
plgtA-ON	Contains <i>lgtA</i> with homopolymeric tract locked ON (<i>G</i> ₁₂ → GGGCGGAGGTGG) by SDM (Quick change Lightning Multi SDM kit; Agilent Tech) with primers lgtA-ON FWD and lgtA-ON REV. Used to generate <i>lgtA</i> locked ON.
plgtA-del	Contains WT <i>lgtA</i> with 417 bp deletion corresponding to bp 50-467 of the coding sequence. The deletion was created by double digesting plgtA with BbsI and SspI and ligating the larger fragment. Used to generate <i>lgtA</i> locked OFF (deletion).
lgtC constructs	
plgtC	Contains wildtype MS11 4/3/1 <i>lgtC</i> amplified with lgtC Fwd-Ext and lgtC Rev-Ext and cloned into pCR2.1 TOPO (Life Technologies, USA). Used to construct plgtC-ON and plgtC-OFF.
plgtC-ES	Contains <i>lgtC</i> with <i>Erm^R-Sm^S</i> from pFLOB4300 inserted into an engineered <i>Sma</i> I site in the homopolymeric tract. Used to construct unmarked <i>lgtC</i> mutations (ON and OFF).
plgtC-ON	Contains <i>lgtC</i> with homopolymeric tract locked ON (<i>G</i> ₁₄ → GGGGCGGAGG) (by SDM [Quick change Lightning Multi SDM kit; Agilent Tech] with primers lgtC-ON FWD and lgtC-ON REV. Used to generate <i>lgtC</i> locked ON.
plgtC-OFF	Contains <i>lgtC</i> with homopolymeric tract locked OFF (<i>G</i> ₁₄ → GGTGAGGGGGGGGG) by SDM (Quick change Lightning Multi SDM kit; Agilent Tech) with primers lgtC-OFF FWD and lgtC-OFF REV. Used to generate <i>lgtC</i> locked OFF.
lgtD constructs	
plgtD	Contains wildtype MS11 4/3/1 <i>lgtD</i> amplified with lgtD Fwd-Ext and lgtD Rev-Ext and cloned into pCR2.1 TOPO (Life Technologies, USA). Used to construct plgtD-del and plgtD-ON.
plgtD-ES	Contains <i>lgtD</i> with <i>Erm^R-Sm^S</i> from pFLOB4300 inserted into an engineered <i>Sma</i> I site in the homopolymeric tract. Used to construct unmarked <i>lgtD</i> mutations (ON and OFF).
plgtD-ON	Contains <i>lgtD</i> with homopolymeric tract locked ON (<i>G</i> ₁₃ → GGGCGGAGGTG) by SDM (Quick change Lightning Multi SDM kit; Agilent Tech) with primers lgtD-ON FWD and lgtD-ON REV. Used to generate <i>lgtD</i> locked ON.
plgtD-del	Contains lgtD with 744 bp deletion corresponding to bp 64-808 of the coding sequence. The deletion was created by digesting plgtD with PfiI and NdeI and ligating the larger fragment. Used to generate <i>lgtD</i> locked OFF (deletion).

All plasmids other than pFLOB4270 were constructed during this study.

Supplemental Table S3: Negative ion MS data and proposed compositions of O-deacylated LPS from *N. gonorrhoeae* strains. (Average mass units were used for calculation of molecular weight based on proposed composition as follows: Hex, 162.15; Hep, 192.17; HexNAc, 203.19; PEtn, 123.05; Kdo, 220.18; Lipid A-OH, 952.00. ¹The relative intensity is expressed as relative to the most populous glycoform/phosphoform.)

Strain	Observed ions (<i>m/z</i>) (M-3H) ³⁻	Observed ions (<i>m/z</i>) (M-2H) ²⁻	Molecular Mass (Da)		Rel. Int. ¹	Proposed Composition
			Observed	Calculated		
5-Hex/G+	997.2	1497.6	2995.9	2993.8	1.0	5Hex, 2HexNAc, 2Hep, 2Kdo, Lipid A-OH
	1065.0	-	3198.0	3197.0	0.4	5Hex, 3HexNAc, 2Hep, 2Kdo, Lipid A-OH
5-Hex/G-	888.9	1333.8	2669.7	2669.6	0.1	3Hex, 2HexNAc, 2Hep, 2Kdo, Lipid A-OH
	930.0	1395.3	2792.8	2792.6	1.0	3Hex, 2HexNAc, 2Hep, 2Kdo, PEtn, Lipid A-OH
	970.8	-	2915.4	2915.6	0.3	3Hex, 2HexNAc, 2Hep, 2Kdo, 2PEtn, Lipid A-OH
	997.8	1496.7	2995.9	2995.7	0.7	3Hex, 3HexNAc, 2Hep, 2Kdo, PEtn, Lipid A-OH
	1039.2	1558.5	3119.8	3118.8	0.2	3Hex, 3HexNAc, 2Hep, 2Kdo, 2PEtn, Lipid A-OH
4-Hex/G+	997.2	1495.8	2994.1	2993.8	1.0	5Hex, 2HexNAc, 2Hep, 2Kdo, Lipid A-OH
	888.9	1333.8	2669.7	2669.6	0.1	3Hex, 2HexNAc, 2Hep, 2Kdo, Lipid A-OH
	930.0	1395.3	2792.8	2792.6	1.0	3Hex, 2HexNAc, 2Hep, 2Kdo, PEtn, Lipid A-OH
3-Hex/G+	875.5	1313.5	2629.3	2630.5	0.4	4Hex, HexNAc, 2Hep, 2Kdo, Lipid A-OH
	930.0	1394.7	2792.2	2792.6	1.0	5Hex, HexNAc, 2Hep, 2Kdo, Lipid A-OH
	983.7	1475.7	2953.8	2954.8	1.0	6Hex, HexNAc, 2Hep, 2Kdo, Lipid A-OH
	-	1496.5	2995.0	2995.8	0.2	5Hex, 2HexNAc, 2Hep, 2Kdo, Lipid A-OH
	1051.5	-	3157.5	3157.9	0.1	6Hex, 2HexNAc, 2Hep, 2Kdo, Lipid A-OH
3-Hex/G-	-	1233.6	2469.2	2468.3	0.5	3Hex, HexNAc, 2Hep, 2Kdo, Lipid A-OH
	864.3	1295.4	2594.4	2591.3	1.0	3Hex, HexNAc, 2Hep, 2Kdo, PEtn, Lipid A-OH
2-Hex/G+	874.8	1313.4	2628.1	2630.4	1.0	4Hex, HexNAc, 2Hep, 2Kdo, Lipid A-OH
2-Hex/G-	808.4	1212.8	2427.9	2429.1	1.0	2Hex, HexNAc, 2Hep, 2Kdo, PEtn, Lipid A-OH



Supplemental Figure S1. C4BP binding to the MS11 *lgtG* 'ON' (G+) mutants. Bacteria were incubated with heat inactivated human complement for 30 min at 37 °C and bound C4BP was detected with anti-human C4BP mAb 67, followed by the biotinylated goat anti-mouse IgG (Molecular Probes), followed by Neuravidin conjugated DyLight 633 (ThermoFisher Scientific). Fluorescence was measured using a BD LSRII. Representative histograms from one of five separate experiments are shown. The number alongside each histogram indicates the median fluorescence intensity of the entire bacterial population.

CHAPTER III

A complement enhancing chimeric antibody confers protection against a hypervirulent strain of *Neisseria gonorrhoeae* and circumvents antigenic variation

Chimeric mAb(s) used in experiments described in this chapter were graciously provided by Genmab B/V, The Netherlands. Drs. Sunita Gulati and Bo Zheng assisted with the animal experiments.

Abstract

Ng, the etiologic agent of gonorrhea has become resistant to multiple antibiotics. Experts have predicted epidemics of untreatable gonorrhea by 2020 unless newer therapeutics are developed. Furthermore, due to lack of broadly conserved immunodominant epitopes and antigenic variation, vaccine development has been a challenge. I have previously published the *in vitro* efficacy of murine mAb 2C7 which targets a LOS epitope seen in over 94% of clinically circulating *Ng*, despite *Ng* LOS glycans being highly phase variable. Thus, this antibody is being developed as a chimeric (2C7-Ximab; murine Fv with human IgG1 C_H1, C_H2, C_H3, and C_L domains) immuno-therapeutic/prophylactic. The susceptibility of persons with complement (C') deficiencies to recurrent disseminated *Ng* infections suggests a role for C' in host defenses against *Ng*. Thus, a 2C7-Ximab variant whose Fc was mutated to enhance Fc hexamerization (E-to-G at position 430) and C1q engagement was evaluated. I observed similar binding patterns of 2C7-Ximab(s) to the four distinct *Ng* LOS structures as with murine mAb 2C7 (2-Hex/G+ >> 4-Hex/G+ ≈ 5-Hex/G+ >> 3-Hex/G+). A ~10-fold lower concentration of 2C7-Ximab with the complement-enhancing Fc mutant was required to achieve similar killing as with wild-type Fc in serum bactericidal assays. While three of four *Ng* LOS mutants (2-Hex/G+, 4-Hex/G+, and 5-Hex/G+) were killed by C'-mediated bacteriolysis, the fourth mutant (3-Hex/G+) that was serum resistant was nevertheless killed (≈ 30% survival) by 2C7-Ximab E430G Fc in the presence of PMNs, 2C7-Ximab E430G

Fc, and C'. *In vivo*, 2C7-Ximab E430G Fc significantly shortened the duration of infection caused by WT *Ng*, 5-Hex/G+, and 4-Hex/G+. Infections with 2-Hex/G+ and 3-Hex/G+ were significantly attenuated (and the Ab did not further decrease infection). These data confirm previous observations in human male volunteers that longer LOS structures (4-Hex/G+ and 5-Hex/G+) are required for durable infection. Thus, 2C7-Ximab represents a promising immunotherapeutic/prophylactic option against multidrug-resistant *Ng* isolates.

Introduction

Gonorrhea is the second most common bacterial sexually transmitted infection with 78.3 million new incidences each year (3). *Neisseria gonorrhoeae* (*Ng* or the gonococcus), the etiologic agent of gonorrhea has become resistant to multiple conventional antibiotics including third generation cephalosporins, macrolides, and tetracyclines (39). Withdrawal of an antibiotic from therapeutic regimens usually does not result in reversal to susceptibility (64). Furthermore, there are no safe and effective vaccines licensed against *Ng*, necessitating an urgent need for novel therapeutics.

Conventional antibiotics target metabolic processes which makes them amenable to bacterial subversion strategies. Antibodies (Ab[s]) physically bind to surface structures or secreted antigens. They either kill bacteria by recruiting host defenses (i.e. complement [C'], phagocytes, cytolytic NK cells) or render them non-infectious by disrupting critical steps in pathogenesis (neutralization). A murine monoclonal antibody (mAb) 2C7 (2C7-murine) reacts with 94% of clinical isolates of *Ng* (87) and shortens the duration and reduces the burden of infection in mice (70). 2C7-murine targets a conserved epitope (Fig. 1) within the gonococcal lipooligosaccharide (LOS) that is critical for pathogenesis (70). Therefore, resistance to mAb 2C7 because of loss of expression of the 2C7 epitope as a result of *lgtG* being phase varied OFF, if it were to occur, would result in considerable loss of bacterial fitness. Thus, mAb 2C7 is being

considered as a therapeutic option against multidrug-resistant *Ng*. As an initial step, we are evaluating a chimeric IgG1 antibody with 2C7-murine Fv (2C7-Ximab) as a therapeutic against *Ng*. The primary objective of this chapter was to demonstrate the *in vivo* and *in vitro* efficacy of 2C7-Ximab against *Ng*.

Binding of 2C7-Ximab to the gonococcal LOS requires a lactose extension from each of the two core heptoses (Hep) I and II (Fig. 1) (139). While the expression of the HepI lactose is constitutive, the extension of the HepII lactose is under the control of the phase-variable (pv) lipooligosaccharide glycosyl transferase G gene (*lgtG*). Deleting *lgtG* reduces fitness of *Ng* in female mice (70). Three other pv genes, *lgtA*, *lgtC*, and *lgtD* contribute to variation of the HepI glycans in the *Ng* LOS (Fig. 1) (195). Of the four HepI structures that can be expressed (2-Hex, 3-Hex, 4-Hex, and 5-Hex), the lacto-N-neotetraose (LNnT or 4-Hex) structure has received the most attention. The terminal galactose in strains expressing 4-Hex from HepI can be sialylated by $\alpha(2,3)$ sialyltransferase that renders *Ng* resistant to killing by complement, at least in part because of enhanced factor H binding (82). Moreover, (unsialylated) LNnT is also thought to play a role in transmission by engaging the asialoglycoprotein receptors (ASGP-R) on spermatozoa (196) and male urethral epithelial cells (32). However, relatively little is known about the role of the other HepI glycans in pathogenesis. Hence, one of the goals of this study was to elucidate how HepI LOS glycans affect colonization *in vivo* in the mouse infection model. As discussed in Chapter II, HepI LOS glycan variations modulate the *in vitro* binding and efficacy of 2C7-murine (122) and all

studies of *in vivo* efficacy of 2C7-murine thus far have been with a wild type relatively avirulent strain of *Ng* (126) predominantly expressing the LNnT/4-Hex LOS structure from its Hepl (70). Consequently, a second objective was to test *in vivo* the efficacy of the 2C7-Ximab that is being considered as a therapeutic against the four 2C7-reactive Hepl LOS glycan mutants (122) in the more infectious (in humans) MS11 background (126).

The Ab initiated classical complement cascade is required for *in vitro* C'-dependent bacteriolysis of *Ng*; the alternative pathway alone is insufficient. C' deficiencies, especially in C6, C7, and C8 were reported in patients with multiple episodes of disseminated gonococcal infections (77, 78) suggesting the importance of C'-mediated host defenses against *N. gonorrhoeae*. The Fc regions of Ab(s) that engage the globular heads of C1q form Fc hexamers when Ab(s) bind to surfaces. Recent advances in Ab engineering have enabled selective upregulation or downregulation of effector functions. Therefore, in collaboration with colleagues at Genmab I asked whether enhancing the C' activating properties of 2C7-Ximab by Fc engineering enhanced its activity *in vitro* as well as *in vivo*. The glutamate (E) at position 430 in the C_H3 domain of IgG1 forms a salt bridge with lysine (K) at position 338 in the C_H2 domain, thereby limiting the flexibility of the C_H2 — C_H3 domains. A E430G mutation abrogates the salt bridge, and allows juxtaposed C_H2 domains to adopt conformations more amenable to hexamer formation (197). The affinity of an individual Fc C_H2 domain for a single globular head of C1q is very low and in the

order of 10^{-4} M (92), but multimerization enhances the avidity of the C1q – Fc interaction (Fig. 2). Alanine scanning mutagenesis has revealed the importance of D270, K322, P329, and P331 residues in Fc in C1q binding (198) (Fig. 2). Therefore, a 2C7-Ximab Null (D270A·K322A) Fc was used as a negative control to elucidate the role of C'-mediated defenses against *Ng* (Fig. 2).

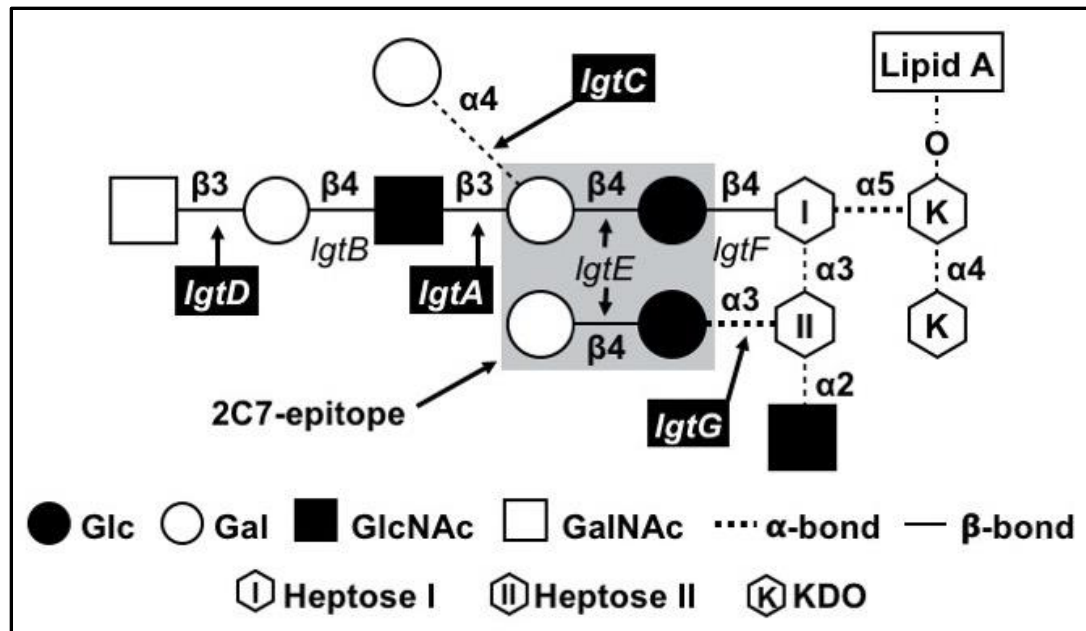


Figure 1. Schematic representation of the phase-variable (pv) *Ng* LOS. *Ng* can express up to eight distinct LOS structures. Variation in the *Ng* LOS is due to pv of four *lipooligosaccharide glycosyltransferase* (*lgt*) genes (indicated within black boxes); *lgtA*, *lgtC*, *lgtD*, and *lgtG*. These genes encode enzymes that catalyze addition of Hex(s) to the Hep(s). Two other *lgt* genes, *lgtF* and *lgtE* are expressed constitutively and the corresponding enzymes add a lactose moiety to HepI. Thus, this is the LOS structure that *Ng* would express when all four pv genes are ‘OFF’. Practically, the expression of *lgtG* (G+) is ‘virtually’ constitutive (in >94% clinical samples), and this results in the addition of a lactose moiety to HepII. The HepI and HepII lactoses together make up the mAb 2C7 epitope (shaded in gray). Depending on the combinations in which *lgtA*, *lgtC*, or *lgtD* are ‘ON’ or ‘OFF’, two (2-), three (3-), four (4-), or five (5-) Hex(s) may be extended from HepI.

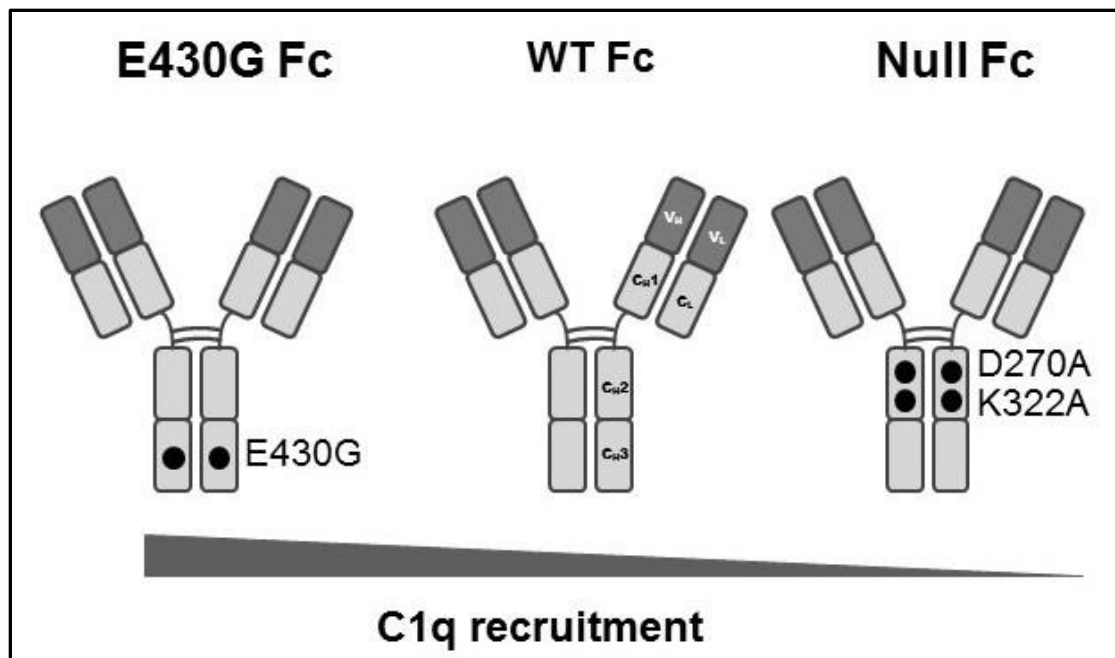


Figure 2. Schematic representation of the three chimeric mAb 2C7(s) (2C7-Ximab[s]). Three 2C7-Ximab(s): E430G Fc, WT Fc, and Null Fc were made by grafting the 2C7-murine Fv (comprising V_H and V_L) region on to a human IgG1 backbone comprising the light and heavy chain constant domains. In the E430G Fc, the glutamate (E) at position was mutated to a glycine (G). E430 forms in the C_H3 domain forms a salt bridge with the lysine, K338 in the C_H2 domain thereby limiting the flexibility of the C_H2 – C_H3 domains. Replacing E with G, a non-polar amino acid abrogates this salt bridge, thereby allowing neighboring C_H2 domains to adopt conformations more amenable to hexamer formation. The globular heads of the hexameric C1q engage Fc regions of Ab(s). Normally, this is a low affinity interaction. Multiple C1q heads simultaneously engaging Fc regions on multiple Ab(s) makes the interaction high avidity and results in stronger complement activation. The WT Fc has the native IgG1 Fc sequence, whereas in the Null Fc the aspartate (D) at 270 as well as the K at 322 have been both mutated to alanine (A). D270 interacts with R129 and K322 interacts with D162 in interactions with the C1q globular head (β-chain) and thus mutating these residues abrogate C1q binding.

Materials and Methods

Bacterial strains and culture conditions

Gonococcal strain MS11 4/3/1, a variant of MS11 VD300 and its isogenic mutants, 2-Hex/G+, 3-Hex/G+, 4-Hex/G+, and 5-Hex/G+ have been described previously (122). The *lgtG* was locked “ON” in MS11 4/3/1 to generate WT-HepI (WT/G+) as described previously (122). Briefly, WT *lgtG* in the streptomycin resistant MS11 4/3/1 was replaced with *lgtG: ErmC rpsL_{F62}* which resulted in transformants that were streptomycin sensitive, but erythromycin resistant. A subsequent transformation in this clone with a plasmid containing the *lgtG*-‘ON’ sequence (122) resulted in homologous recombination and replacement of *ErmC rpsL_{F62}* with *lgtG*-ON; similar to the wild-type strains, this mutant was also streptomycin resistant and erythromycin sensitive (122).

Ng from freezer stocks were streaked on chocolate agar (CA) plates with supplemented Isovitallex and incubated overnight (~18 h). For *in vivo* experiments, *Ng* were cultured on CA supplemented with vancomycin (V), colistin (C), nystatin (N), trimethoprim sulfate (TS), and Isovitallex (CA-VCNTS agar). Bacteria harvested from overnight cultures were sub-cultured onto fresh CA plates for 6 h at 37 °C in the presence of 5% CO₂ for *in vivo* experiments. For *in vitro* assays, overnight cultures on CA plates were suspended in liquid media comprising Morse A and Morse B and Isovitallex for 4 hours at 37 °C with shaking at 250 rpm.

Construction, expression and purification of chimeric mAb 2C7 Ab(s)

Three 2C7-Ximab(s) were generated by Genmab BV, The Netherlands. A schematic of the mAb(s) is provided in Fig. 2. The mAb(s) were produced in Chinese Hamster Ovary cells using Genmab's proprietary methods.

Human complement

Human C' was prepared as described previously (122). Briefly, normal human serum that contained NaCl 1M and 10 mM EDTA was depleted of endogenous IgG and IgM by passage over protein A/G (Pierce, USA) and anti-human IgM (Sigma) columns, respectively. Subsequently, the eluates were spin concentrated, equilibrated against PBS/0.1 mM EDTA and filter sterilized. All steps were carried out at 4 °C. Aliquots were stored at -80 °C, and thawed before use.

2C7-Ximab binding assays

The four HepI LOS mutants (10^7 CFU each) were incubated with 10, 31, or 100 µg/mL of each of the three 2C7-Ximab(s) for 30 min at room temperature with shaking at 150 rpm. Bound Ximab was detected with FITC conjugated anti-human IgG (Sigma) for 15 min. Fluorescence was measured by flow cytometry (FCM) using a LSRII (BD Biosciences, Franklin Lakes, NJ). FCM data were analyzed using FlowJo (v 10.2, Tree Star, Ashland, OR).

Serum bactericidal assays

Serum bactericidal assays were performed as described previously (122). About 2000 CFU of bacteria were incubated at 37 °C (with 5% CO₂) in the presence of C' and/or Ab(s). Aliquots were plated at 0 and 30 min and percent survival was calculated by expressing the number of CFU at 30 min as a percent of the number of CFU at 0 min. C1q depleted sera and purified C1q were purchased from Complement Technology, Inc., USA.

PMN killing and association assays

PMN killing assays were performed as previously described (122). Briefly, PMNs were isolated from freshly drawn venous blood over a Percoll gradient (density 1.130 g/mL; GE Lifesciences) after initial separation over Histopaque K119 (Sigma). PMNs were then adhered on to tissue culture coated plastic cover slips (Sarstedt, Germany) following which they were infected with either opsonized or un-opsonized *Ng* at a MOI of 1. Aliquots were plated at 0 and 60 min and percent survival was calculated by expressing the number of CFU at 60 min as a percent of the number of CFU at 0 min. For association assays, adherent PMNs were infected with Hoechst 33342 labeled bacteria at a MOI of 10. Adherent PMNs were scraped off after an hour, washed and analyzed by FCM over a BD LSRII. Un-opsonized bacteria and non-infected PMNs were used as controls.

Complement C1q binding and C3 deposition assays

Approximately 10^7 CFU of *Ng* were incubated (in the presence of absence of 2C7-Ximab[s]) either with 20% C' (for C3 deposition) or with 3 μ g of C1q (for C1q binding) for 15 min. C3 deposited on bacteria was stained with FITC conjugated sheep anti-human C3c (Bio-Rad) for 15 min. Paraformaldehyde (2%) was added to reactions and incubated for 10 min at 37 °C. C1q associated with bacteria was detected with biotinylated anti-C1q (JL-1 clone, Abcam) for 30 min, followed by streptavidin – DyLight633 (Thermo Fischer) for 15 min.

In vivo infection studies

Six to eight weeks old female BALB/c mice (Jackson Laboratories) were infected with *Ng* as described previously (70). Briefly, mice were selected in the diestrus phase of estrous cycle and administered 0.1 mg Premarin (Pfizer) per day subcutaneously as a single dose on days -2, 0, and +2. Mice were also given two daily intra-peritoneal doses of 0.6 mg of vancomycin (Mylan pharmaceuticals) and 0.3 mg of streptomycin (X-gen pharmaceuticals) on days -2, -1, and 0. Premarin, streptomycin, and vancomycin were all diluted in sterile water. On day 0, mice were infected intra-vaginally with 10^6 CFU of one of the five *Ng* mutants: WT/G+, 5-Hex/G+, 4-Hex/G+, 3-Hex/G+, or 2-Hex/G+. Animals in the treated groups received 1 or 0.1 μ g of 2C7-Ximab E430G Fc in saline intra-vaginally daily beginning on day 0. Control groups were administered saline. Vaginal swabs were collected daily for 9 days; bacteria in swabs were eluted in 100 μ L

saline. Bacterial burdens were monitored by quantitative cultures of vaginal swab eluates on CA-VCNTS agar. Mice were declared to have cleared the infection after three successive negative cultures.

Statistical analyses

One-way ANOVA was employed to compare multiple groups using Tukey's post-hoc test for pair-wise comparisons. Two-way ANOVA was used to compare groups when concentration was a variable. *In vivo* clearance experiments were tested for: time to clearance, longitudinal trends in mean log₁₀ CFU, and the cumulative CFU (area under the curve [AUC]). Kaplan-Meier survival curves yielded the median times to clearance; Mantel-Cox log-rank test was used to compare times to clearance between groups. Significance was set using Bonferroni's correction for number of pairwise comparisons when more than two groups were compared – for example, a significant P value when three groups were compared was set at 0.017 (0.05/number of possible pairwise comparisons, which is 3). Bacterial burdens were determined for each group by averaging the area under the curve (log₁₀CFU vs. time) for each mouse. Bacterial burdens were then compared using ANOVA (Kruskal-Wallis test).

Results

Point mutations in the Fc region(s) of 2C7-Ximab(s) do not alter binding to Hepl LOS mutants; Hepl LOS glycans modulate 2C7-Ximab binding

Binding of the three 2C7-Ximab(s), WT Fc, E430G Fc, and Null Fc to the previously described (122) *Ng* Hepl LOS mutants was measured by FCM using concentrations of 10, 31, and 100 µg/mL. Each Hepl mutant bound similar amounts of all three 2C7-Ximab(s) (Fig. 3). However, the amount of antibody binding varied across the four Hepl mutants. Maximum 2C7-Ximab binding was observed with the 2-Hex/G+ mutant followed by the 4- and the 5-Hex/G+, while the 3-Hex/G+ bound the least (Fig. 3). These binding patterns were consistent with those observed previously with 2C7-murine (122). At concentrations below 10 µg/mL, the 2-Hex/G+ was the only mutant that showed binding above control values (dotted lines in Fig. 3); binding to the other mutants above control values was not discernable (data not shown).

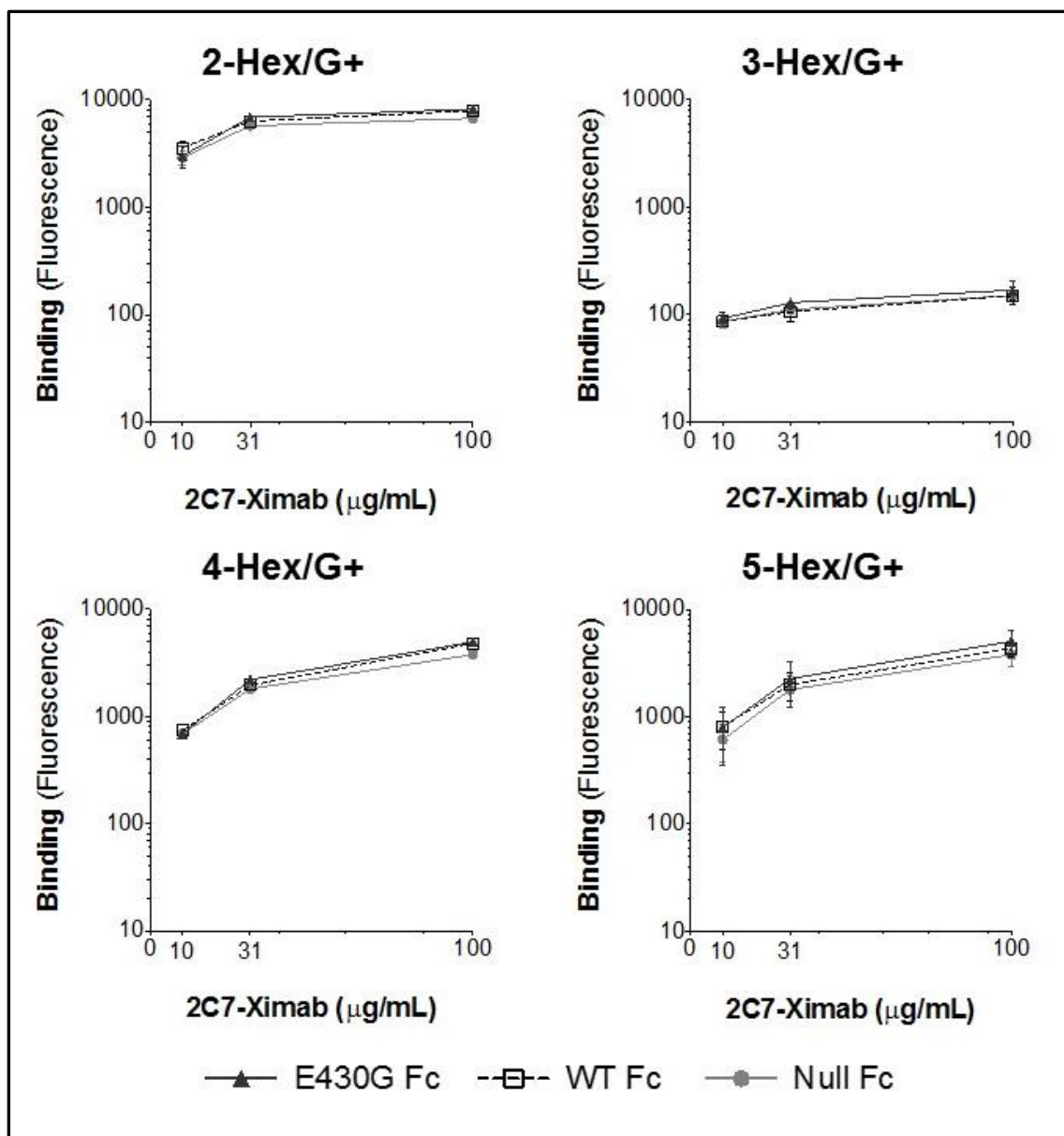


Figure 3. All three 2C7-Ximab(s): E430G Fc, WT Fc, and Null Fc show similar amounts of binding to all four HepI LOS mutants containing the 2C7-epitope. Among the four mutants, maximum binding was observed to the 2-Hex/G+ mutant, followed by the 4-Hex/G+ and 5-Hex/G+ mutants. The 3-Hex/G+ mutant shows the least amount of 2C7-Ximab binding. The dashed line in each graph represents the fluorescence levels of the conjugate control. Each data point represents the mean fluorescence observed in 3 replicate experiments ($\pm\text{SEM}$).

A E430G mutation in the Fc region of 2C7-Ximab enhances serum bactericidal efficacy against Ng

The E430G mutation was originally described for anti-CD38 or anti-CD20 Ab(s) targeting cells of the B-cell lineage (198) with diameters ranging from 5 to >12 μm . In comparison, the average diameter of *Ng* is about 1 μm and 2C7-Ximab targets an epitope within the most abundant molecule on the Gram-negative outer membrane, the LOS. Hence, we speculated that the spatial distribution of WT Fc may already favor optimal C' activation, and E430G Fc may confer no added benefit. To test this hypothesis, we performed serum bactericidal assays on the 2-Hex/G+ mutant with 20% human C' (IgG and IgM depleted normal human serum) and 10, 31, 100, or 316 $\mu\text{g/mL}$ of E430G Fc and WT Fc. The 2-Hex/G+ mutant was chosen because this mutant bound the maximum amounts of 2C7-Ximab. Survival was represented by comparing the number of CFU at 30 mins compared to the number of CFU at 0 mins. Contrary to my expectations, the E430G Fc showed significantly more bactericidal activity than the WT Fc at 31 and 100 $\mu\text{g/mL}$, thereby showing a positive impact of Ab hexamerization on C' activation and *Ng* killing (Fig. 4a). As expected, the Null Fc which is engineered to abrogate C1q-Fc interactions did not cause any C' dependent bacteriolysis (survival >100%) even at 316 $\mu\text{g/mL}$ (Fig. 4b).

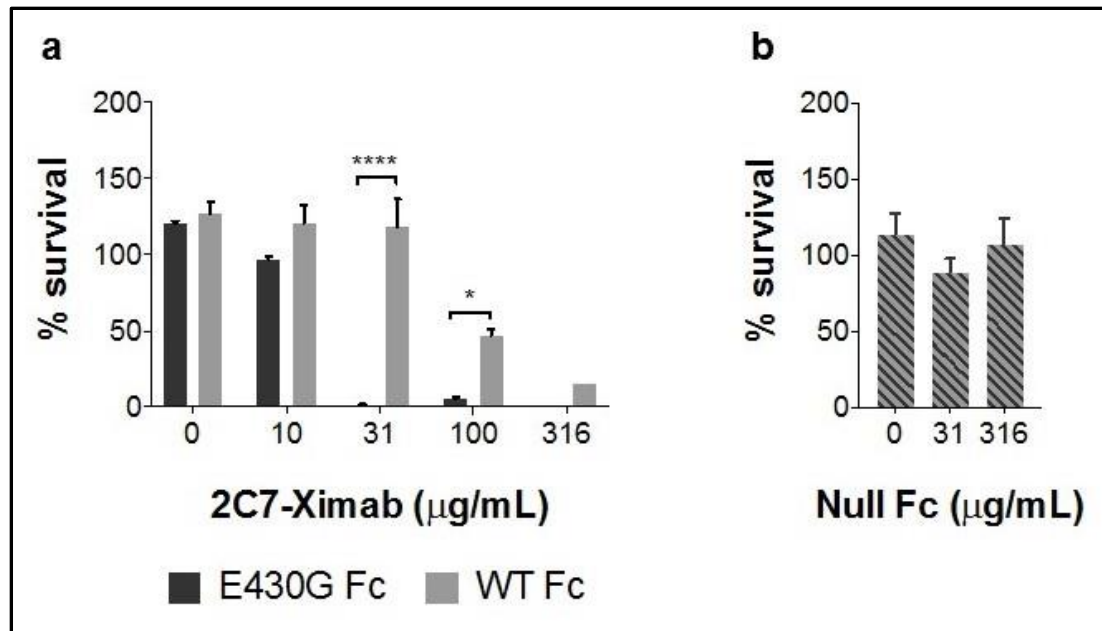


Figure 4. Serum susceptibility of the 2-Hex/G+ mutant in the presence of 2C7-Ximab(s). **(a)** 2C7-Ximab E430G Fc is bactericidal for the 2-Hex/G+ mutant at a log₁₀-fold lesser concentration compared to WT Fc. Each bar represents the mean of 2 replicates (\pm range). Groups were compared using 2-way ANOVA (*, $P = 0.0133$; ****, $P = <0.0001$). **(b)** The Null Fc does not have any cidal activity. Survival was calculated by expressing the number of colonies at T=30 min as a percent of the number of colonies at 0 min.

The enhanced bactericidal activity of 2C7-Ximab E430G Fc is associated with increased C1q binding

To confirm that the increased bactericidal activity of the E430G Fc was indeed due to the C1q recruitment enhancing property of the E430G mutation (92, 197), I measured C1q deposition on the 2-Hex/G+ mutant in the presence of 31, 56, or 100 µg/mL of each of the three 2C7-Ximab(s) by FCM. In every instance, I observed significantly more C1q recruitment by the E430G Fc compared to the WT Fc. The WT Fc recruited more C1q than the Null Fc (significant at 56 and 100 µg/ml). Minimal C1q recruitment was noted in the absence of 2C7-Ximab (Fig. 5a and Suppl. Fig. 1). No killing of *Ng* was observed when E430G opsonized bacteria were incubated with C1q depleted serum; the addition of purified C1q reconstituted the bactericidal activity of serum (Fig. 5b). Collectively, these observations suggest that the enhanced bactericidal activity of E430G Fc is directly linked to its ability to increase C1q recruitment and classical C' pathway activation.

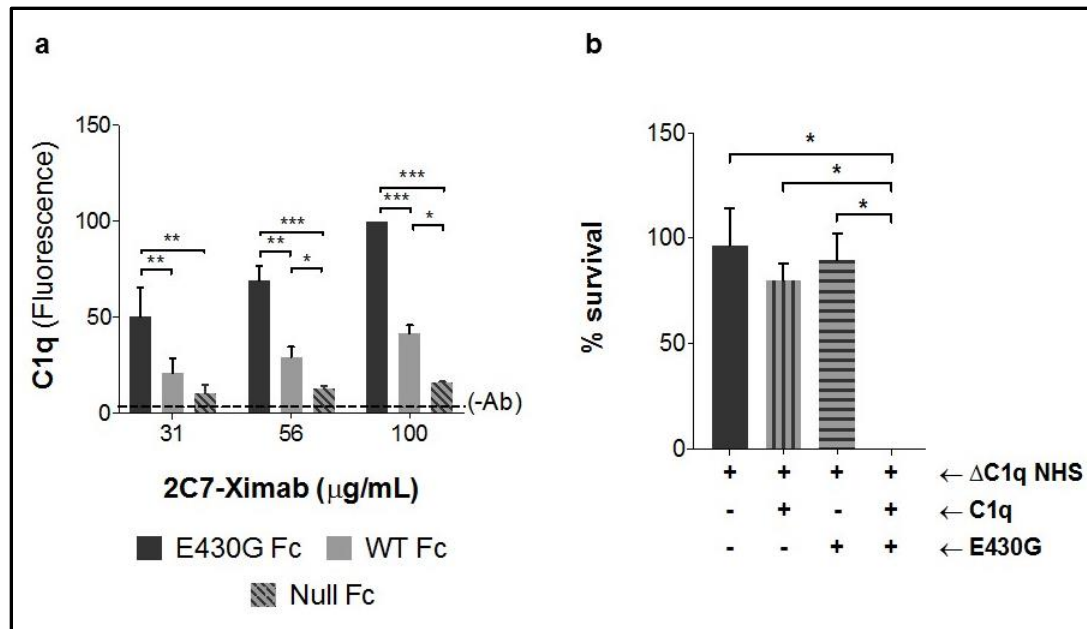


Figure 5. Enhanced C1q recruitment is necessary for the enhanced bactericidal efficacy of 2C7-Ximab E430G Fc. **(a)** E430G Fc binds significantly more C1q than WT Fc on the 2-Hex/G+ mutant at each tested concentration. Each bar represents the mean of 2 replicates (\pm range). Comparisons across groups was made using 2-way ANOVA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). **(b)** C1q is necessary for bactericidal activity of E430G. Serum bactericidal assays with the 2-Hex/G+ mutant was performed with 20% C1q depleted NHS (Δ C1q NHS) or Δ C1q NHS reconstituted with pure C1q (70 μ g/ml). Each bar represents the mean of 2 replicates (\pm range). Comparison between groups were made using one-way ANOVA. Overall P value was 0.0112 (*, $P < 0.05$).

The ability of 2C7-Ximab E430G Fc to activate the classical C' cascade is regulated by Ng Hepl LOS glycans

Next, I tested the bactericidal activity of E430G Fc against the three other Hepl LOS mutants. Serum bactericidal assays were performed with 20% C' and 31, 100, or 178 µg/mL of E430G Fc. As shown in Fig. 4a, only the 2-Hex/G+ mutant was sensitive to the bactericidal effects of E430G Fc at 31 µg/mL, while the 4-Hex/G+ and the 5-Hex/G+ mutants were killed (<50% survival) in the presence of 100 µg/mL and 178 µg/mL of E430G Fc respectively (Fig. 6). The 3-Hex/G+ mutants remained fully serum resistant (>100% survival) at 178 µg/mL; further increasing mAb concentrations to 500 µg/mL caused no killing (data not shown). Thus, the serum bactericidal assays were consistent with 2C7-Ximab binding patterns. Sensitivity to C' in presence of 2C7-Ximab mimicked observations with 2C7-murine (122).

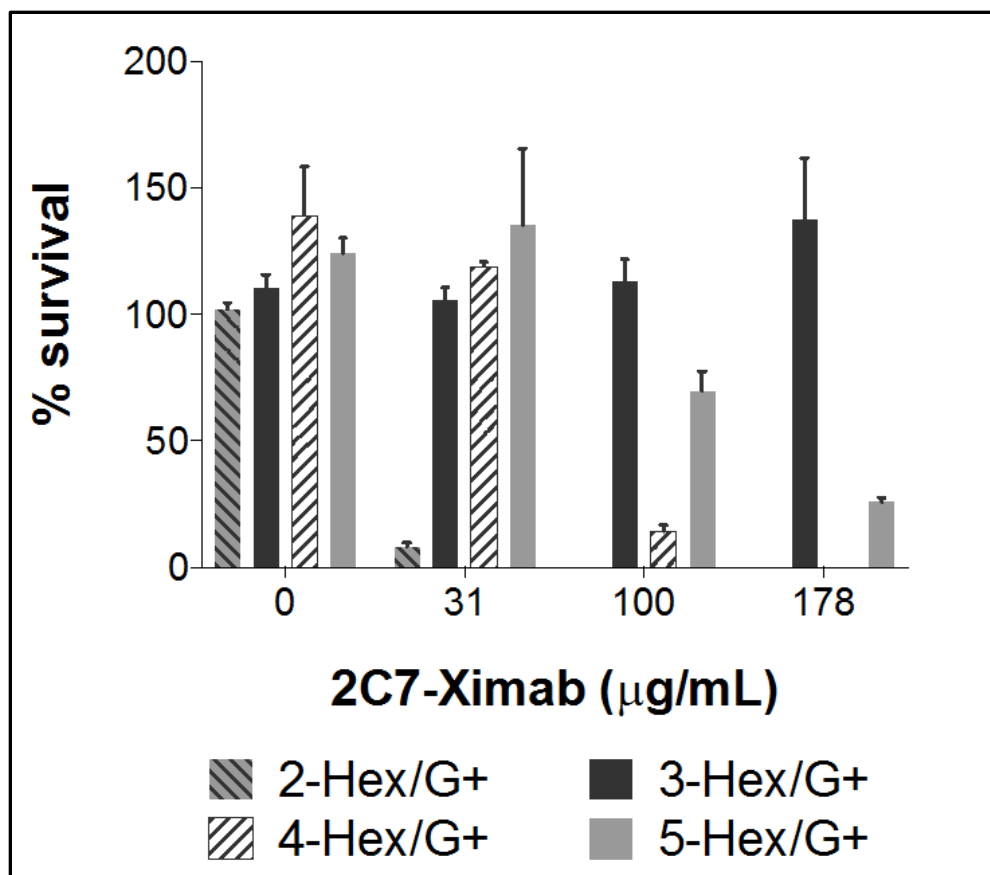


Figure 6. Bactericidal efficacy of 2C7-Ximab E430G Fc follows binding patterns. Serum bactericidal assays were done with 20% human C' as described previously. Each bar represents the mean of 2 replicates (\pm range).

3-Hex/G+ is killed by PMNs in a complement dependent manner upon opsonization with 2C7-Ximab(s)

Our previous studies demonstrate that even though the 3-Hex/G+ mutant was resistant to direct C'-mediated bacteriolysis, C' activation mediated by 2C7-murine opsonization was sufficient to facilitate killing by PMNs (122). Therefore, to assess if 2C7-Ximab(s) also could activate the classical cascade on 3-Hex/G+, we measured C3 deposition by FCM. Both E430G Fc and WT Fc deposited C3 on the 3-Hex/G+ mutant. Although not statistically significant, there was a trend toward higher C3 deposition by the E430G Fc at both 100 and 200 µg/mL compared to the WT Fc (Fig. 7a). Next, I investigated whether opsonization with 2C7-Ximab, either alone or in conjunction with C' results in killing of the 3-Hex/G+ mutant by freshly isolated human PMNs. To assess the effects mediated only by Ab and C', the 3-Hex/G+ mutant was made in MS11 where all 11 Opa proteins were deleted (MS11 Δ Opa; kindly provided by Dr. Daniel C. Stein, University of Maryland; (168)) as CEACAM3 on PMNs can bind to Opa(s) and mediate non-opsonic phagocytic killing. Survival was calculated by expressing the number of CFU at 60 min as a percent of the number of CFU at 0 min. PMNs failed to kill bacteria opsonized with either of the 2C7-Ximab(s) alone at 100 µg/mL. However, a significant decrease in survival was observed when bacteria were incubated with PMNs in the presence of E430G Fc and C'. In contrast, the WT Fc plus complement did not significantly reduce survival (Fig. 7b).

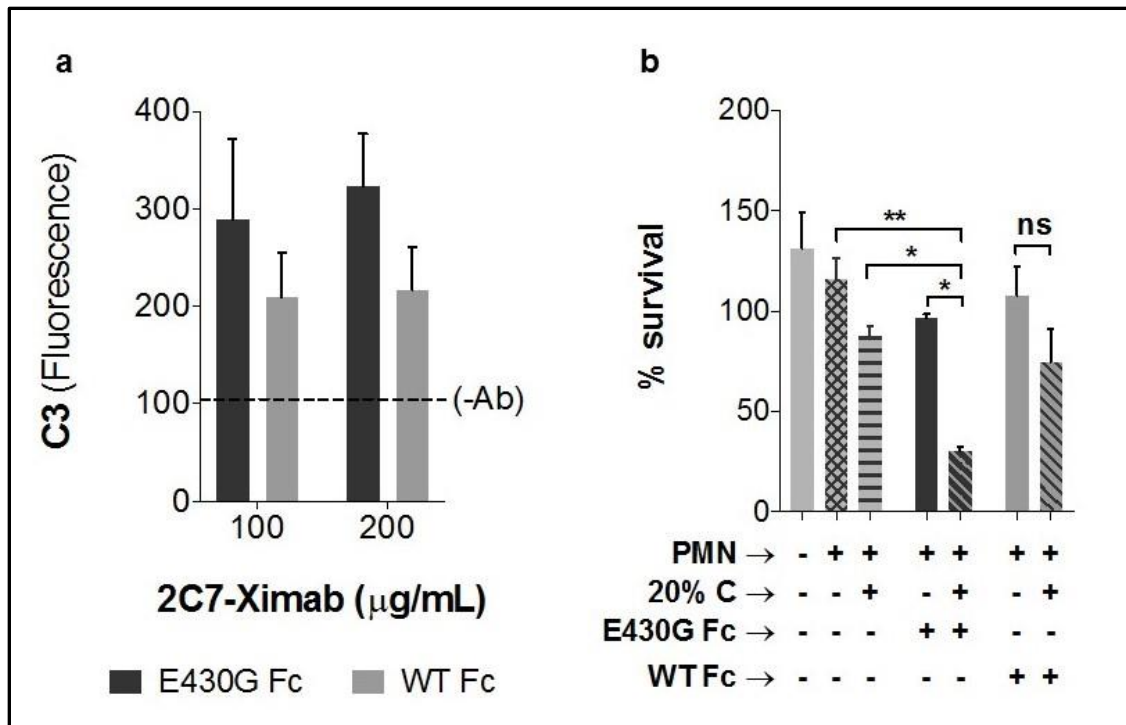


Figure 7. 2C7-Ximab E430G Fc deposits more C3 on the 3-Hex/G+ mutant compared to WT Fc and mediates killing by PMNs. **(a)** C3 deposition on the 3-Hex/G+ mutant. Control reactions included bacteria incubated with 20% C' alone and are shown by the dashed line. The geometric mean of the fluorescence is represented (mean of 2 replicates \pm range). Groups were compared using 2-way ANOVA; differences between E430G Fc and WT Fc were not significant. **(b)** 2C7-Ximab E430G Fc facilitates opsonophagocytosis of 3-Hex/G+ by PMNs in the presence of C'. Percent survival (CFU at 60 min relative to CFU at 0 min) is shown on the Y-axis. Each bar represents the mean of the percent survival of 3 separate experiments using PMNs from a single donor (\pm range). A comparison of killing across the four groups that contained PMNs was performed by one-way ANOVA, using Tukey's multiple comparison test (Overall P value = 0.0007; *, P < 0.05, **, P < 0.01). The strain used in these assays lacked all 11 Opa proteins thus reducing noise from Opa – CEACAM interactions.

The E430G Fc mutation does not enhance association with human PMNs

The data in Fig. 7b show that Opa-negative *Ng* are not killed when PMNs are incubated with *Ng* opsonized with either WT Fc or E430G Fc alone. I next asked if the E430G mutation affected association of Opa-negative *Ng* with human PMNs. Therefore, I assessed the association of fluorescently labelled bacteria with freshly isolated human PMNs in the presence or absence of 100 µg/mL of each of the three 2C7-Ximab(s) to look for differences in FcγR engagement. To reduce background signal because of Opa-CEACAM interactions, we used a MS 11 Δ Opa mutant with a WT LOS. Opsonization with the 2C7-Ximab(s) enhanced the association of bacteria with PMNs; only E430G Fc and WT Fc significantly enhanced association of bacteria with PMNs over baseline levels (no added mAb) levels. I speculate that the increased association compared to non-opsonized bacteria is because of FcγR engagement (Fig. 8a). Although association of *Ng* with PMNs was higher with the E430G Fc and WT Fc when compared to association seen with the Null Fc, these differences were not statistically significant. As shown in Fig. 7b, enhanced association of bacteria with PMNs did not translate to enhanced killing at 60 min as there were no differences in survival between non-opsonized bacteria and *Ng* opsonized with 100 µg/mL 2C7-Ximab in the presence of PMNs (Fig. 8b). Note that the presence of PMNs alone (no added Ab) resulted in a decrease in survival compared to bacteria in media alone (no PMNs or Ab).

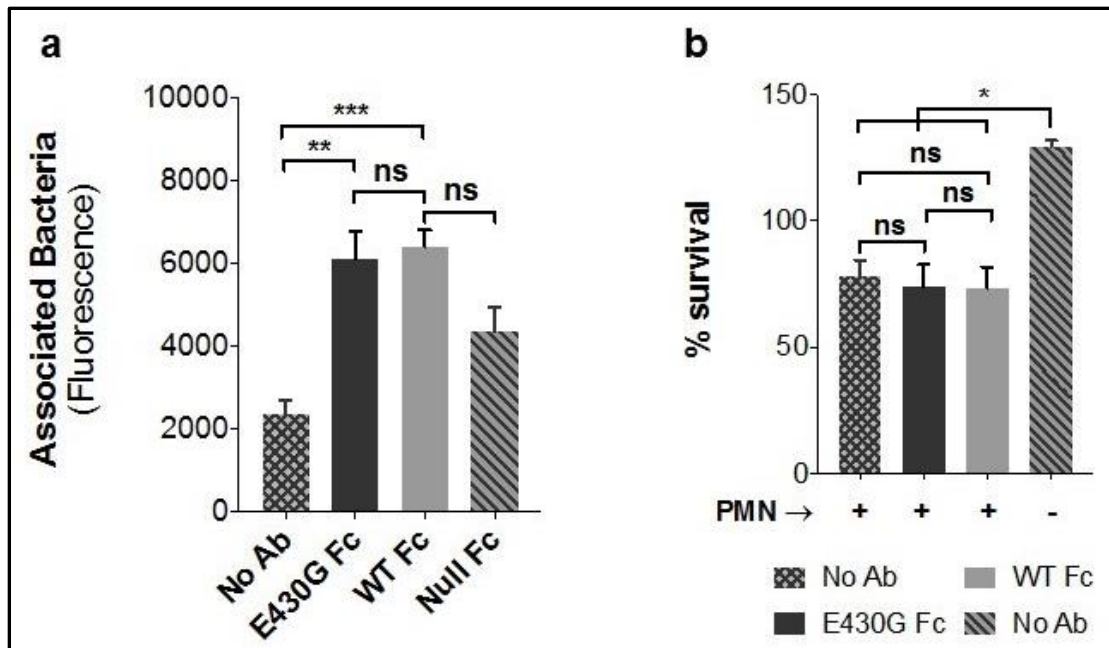


Figure 8. Opsonization of MS11 ΔOpa with 2C7-Ximab increases association with, but not killing by PMNs. **(a)** Opsonization with 2C7-Ximab(s) results in enhanced association of *Ng* with PMNs. Adherent PMNs were infected with Hoechst 33342 labeled bacteria at a MOI of 10. Fluorescently labeled bacteria were opsonized with 100 $\mu\text{g/mL}$ of each of the three 2C7-Ximab(s). Un-opsonized bacteria and non-infected PMNs were used as controls. Groups were compared using one-way ANOVA (**, $P < 0.01$; ***, $P < 0.001$). **(b)** Enhanced association of 2C7-Ximab opsonized bacteria does not result in enhanced killing by PMNs. In killing assays, no difference in survival was noted between opsonized or un-opsonized bacteria exposed to PMNs. Groups were compared by ordinary one-way ANOVA (*, $P = 0.05$).

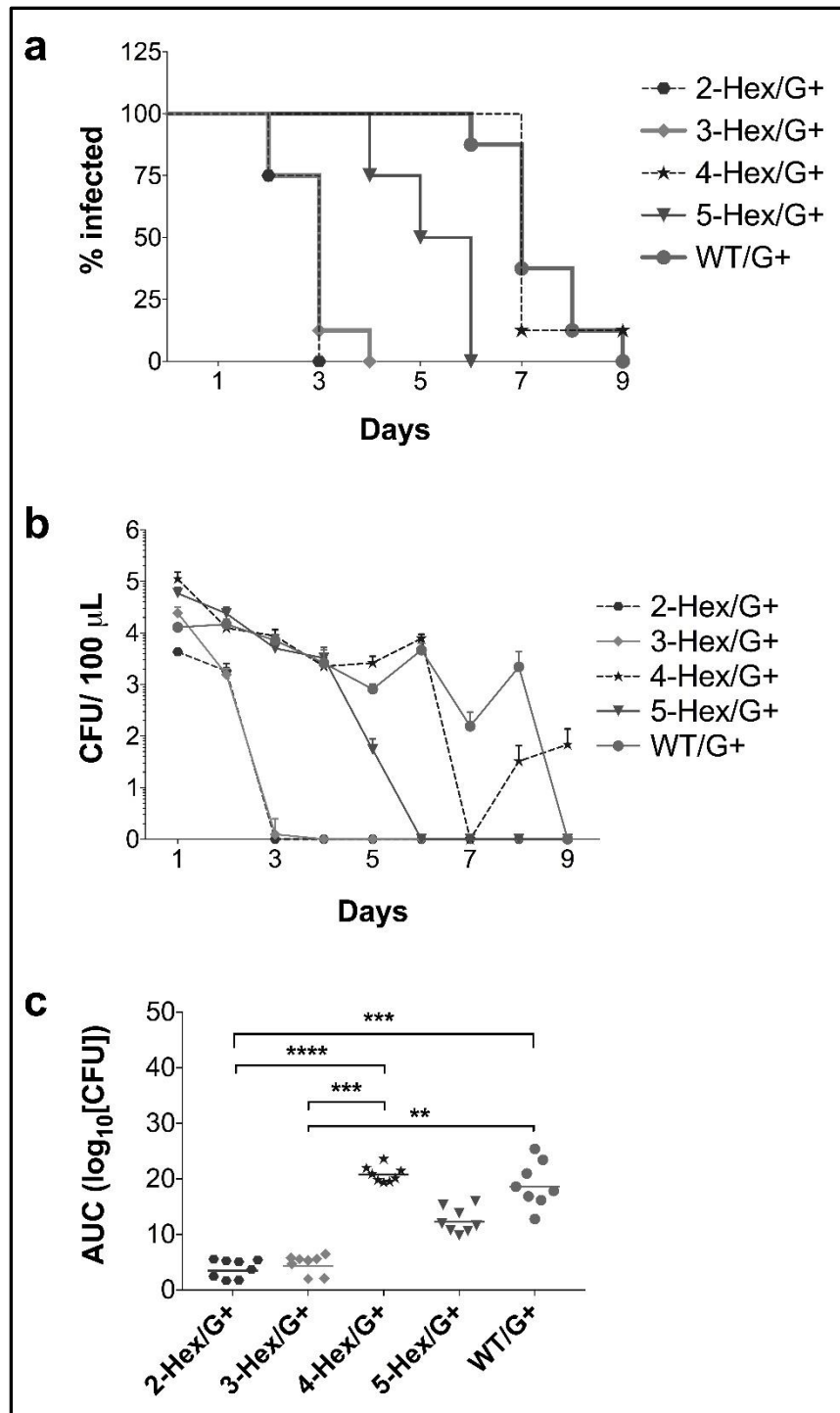
All Hepl LOS mutants colonize mice, but longer Hepl glycans are required for prolonged infection

Hepl glycan structures affect pathogenesis and virulence in the human male volunteer infection model of gonorrhea (125, 199). I aimed to evaluate the effects of Hepl glycan extensions on the virulence of *Ng* in the mouse vaginal colonization model using the four mutants with *IgtG* locked ON (G+). Only the G+ mutants were chosen because loss of *IgtG* expression significantly attenuates infection in mice (70, 89). I included a mutant that had only *IgtG* locked on (WT/G+), which unlike the other four mutants, permitted Hepl phase variation. This mutant would also provide information about a role for Hepl phase variation, if any, in pathogenesis.

Mice were infected with 10^6 CFU of *Ng* intra-vaginally. Vaginas were swabbed and cultured daily to monitor the burden of infection. The 4-Hex/G+ and WT/G+ were the most infective and both these mutants caused similar durations (median time to clearance was 7 d; Fig. 9a) of infection, CFU over time (Fig. 9b) and overall burdens of infection (as measured by AUC; Fig 9c). The 5-Hex/G+ which in addition also expresses the sialylatable L_NnT epitope (122) showed intermediate infectivity (median time to clearance 5.5 days). The 2- and the 3-Hex/G+ mutants infected mice only transiently (median times to clearance 3 days; Fig. 9a) and had a significantly lower overall burden of infection (Fig. 9c). Times to clearance of infection were compared (two groups at a time) using the

Mantel-Cox log-rank test and results are indicated in Table 1. Significance was set at 0.005 (Bonferroni's correction for 10 possible pairwise comparisons). Our results show that while shorter Hepl glycans (i.e., 2- and 3-Hex/G+) can colonize mice, the infection is transient with low overall bacterial burdens (Fig. 9). Longer and more robust infection is favored by longer Hepl glycans, possibly because of expression of the sialylatable LNT LOS, which is consistent with prior observations in mice and men (125, 199).

Figure 9. Longer Hepl glycans are required for prolonged and robust infections *in vivo*. **(a)** Mutants with 4 or 5 Hex(s) extending from Hepl cause prolonged infections in mice. WT/G+ has a predominantly 4-Hex/G+ phenotype (data not shown). Median times to clearance for the 4-Hex/G+ and WT/G+ mutants were 7d. The median time to clearance was 5.5 d for the 5-Hex/G+ mutant, whereas median times to clearance for the 2-Hex/G+ and 3-Hex/G+ mutants were 3 d. Kaplan-Maier (KM) curves were plotted to determine median times to clearance. Mutants were compared pairwise using the Mantel-



Hex/G+ mutants were 3 d. Kaplan-Maier (KM) curves were plotted to determine median times to clearance. Mutants were compared pairwise using the Mantel-

Cox log rank test to determine differences in clearance times indicated in Table 1. **(b)** Mutants with 4 or 5 Hex(s) extending from Hepl cause infections with higher bacterial burdens. The number of colonies recovered from all animals infected with a particular mutant were averaged (\pm SEM) and plotted against time. **(c)** Mutants with longer Hepl LOS glycans cause more robust infections compared to mutants with shorter Hepl glycan extensions. The AUC (\log_{10} CFU) for each mouse is plotted. Horizontal lines represent the geometric means of AUCs for each group. Groups were compared using one-way ANOVA (Kruskal-Wallis) test (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

Table 1: Comparison of median times to clearance of infection (Mantel-Cox) log-rank test				
	2-Hex/G+	3-Hex/G+	4-Hex/G+	5-Hex/G+
3-Hex/G+	ns			
4-Hex/G+	0.0002	<0.0001		
5-Hex/G+	0.0002	0.0004	<0.0001	
WT/G+	0.0002	<0.0001	ns	<0.0001

E430G 2C7-Ximab shortens duration of colonization in mice infected with 'virulent' mutants

Finally, we asked if the E430G Fc (the 2C7-Ximab with superior *in vitro* activity) was effective *in vivo*. Ongoing work in our group has demonstrated that activation of C' is indeed important for clearance of *Ng* infections using two approaches. First, 2C7-Ximab Null Fc fails to clear infections and second, 2C7-Ximab E430G is ineffective in C1q knockout mice (Sunita Gulati; personal communication, 2017). Mice infected with HepI LOS mutants were administered either 1 or 0.1 μ g E430G Fc daily, and CFU recovered from treated mice were compared to those from untreated mice to determine efficacy of the immunotherapeutic. Note that the control arm (no mAb) for this experiment is the same as shown in Fig. 9; the data have been shown separately for ease of interpretation. For the 2-Hex/G+ and 3-Hex/G+ mutants, the median times to clearance of infection in untreated mice (3 days) were similar to mice treated with E430G Fc (2.5 – 3 days) (Fig. 10a and 10b). Bacterial burdens between the treated and untreated groups were also similar (Suppl. Fig. 4a and 4b). Hence, therapy did not confer any added benefit in these attenuated mutants. For the 4-Hex/G+, 5-Hex/G+ and WT/G+, the median times to clearance in mice treated with 1ug/day of E430G Fc were 2 – 2.5 days, whereas that in mice treated with 0.1ug/day they were 2.5 – 3 days (Fig. 11a, 11b, and 11c). The median times to clearance in untreated mice were 7 days for the 4-Hex/G+ and the WT/G+ mutants, and 5.5 days for the 5-Hex/G+ mutant. For these three mutants, the bacterial burdens in the treated groups

were also significantly lower compared to the untreated groups (Suppl. Fig. 5a, 5b, and 5c). The higher dose of 1ug/day of E430G Fc did not provide any added benefit compared to 0.1ug/day.

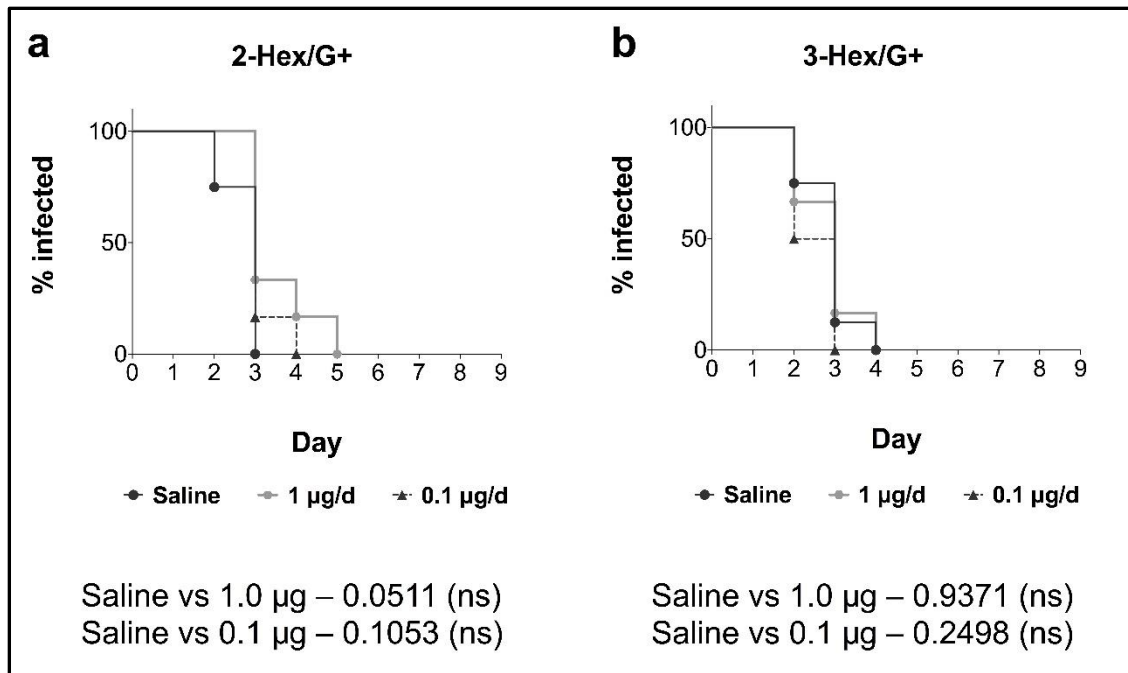


Figure 10. Treatment with 2C7-Ximab E430G does not further attenuate infection by the 2-Hex/G+ or 3-Hex/G+ mutants. **(a)** Treatment does not significantly shorten infection in mice infected with 2-Hex/G+ mutants. **(b)** Treatment does not significantly shorten infection in mice infected with 3-Hex/G+ mutants. Treated mice received 1 or 0.1 µg of 2C7-Ximab E430G Fc in saline intra-vaginally everyday starting on day 0. Control groups were administered saline. KM curves were compared as described previously.

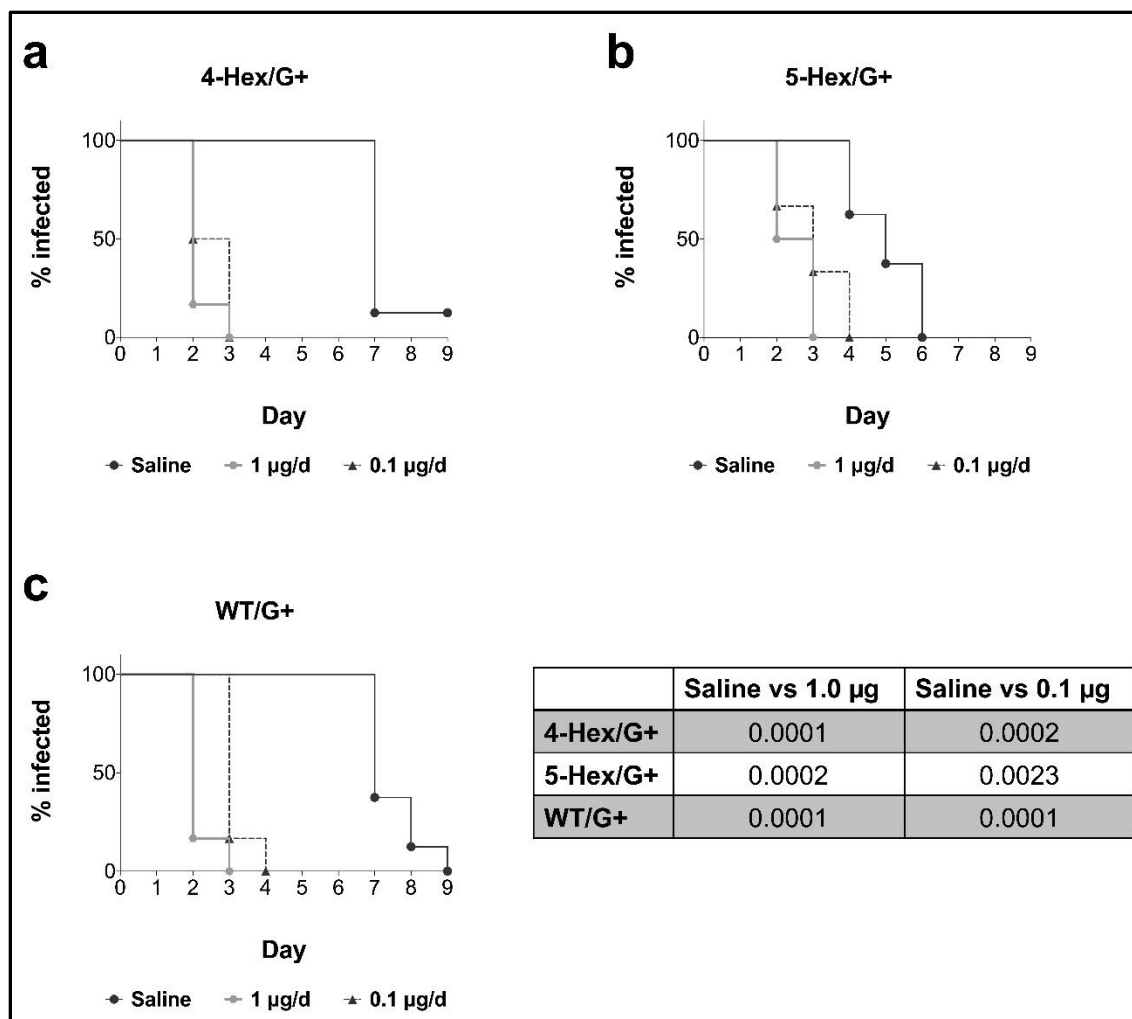


Figure 11. 2C7-Ximab E430G Fc shortens infection in mice infected with 4-Hex/G+, 5-Hex/G+, and WT/G+ mutants. Treatment of mice with 1.0 or 0.1 µg intra-vaginally daily significantly shortens the duration of infection with 4-Hex/G+ **(a)**, 5-Hex/G+ **(b)** and WT/G+ **(c)** mutants compared to control saline treated animals. Significance is set at 0.017 (Bonferroni's correction for three pairwise comparisons in each group).

Discussion

Ng has become multidrug resistant. Strains resistant to even third generation cephalosporins such as ceftriaxone have been identified worldwide (37, 39, 64, 131), and it has been suggested that by 2020 untreatable strains of gonorrhea could be widespread (39, 64). Prior work by our group had identified a monoclonal antibody (2C7-murine) that reacted with > 94% of circulating strains of *Ng* (87, 122). Therefore, this mAb has the potential to be developed as an immunotherapeutic (or immunoprophylactic), which led to developing the 2C7-Ximab(s) in collaboration with colleagues at Genmab. Since C' deficiencies have been associated with a predisposition to recurrent *Neisserial* infections (77, 78, 90), we reasoned that the introduction of C' enhancing properties (the E430G mutation) in the Fc region of 2C7-Ximab would render it more effective. This is especially important since *Ng* possesses multiple mechanisms to escape the bactericidal effects of C', including binding of the C' inhibitors, FH and C4BP (200, 201).

The *in vitro* findings reported here support the greater potency of 2C7-Ximab E430G Fc over 2C7-Ximab WT Fc. Similar to our observations with 2C7-murine, Hep1 LOS glycans modulated the binding and functional efficacy of all 2C7-Ximab(s). A comparison between the E430G Fc and WT Fc revealed that E430G Fc not only promotes direct C' mediated bacteriolysis, but also C' dependent PMN mediated killing at lower doses than the WT Fc (Ab opsonization alone in

the absence of C' results in association of bacteria with PMNs, but does not cause killing). Further, in the murine vaginal colonization model, we demonstrated the ability of E430G Fc to clear infections by *Ng* mutants with two (4-Hex/G+ and 5-Hex/G+) of the four HepI LOS phenotypes associated with the relevant IgtA/C/E phase variation combinations. An important observation with the uninfected controls was that mutants with longer (4-Hex) HepI LOS structures resulted in durable infection. The 5-Hex mutant also expresses the 4-Hex structure, which is modified by sialic acid *in vivo* (177), a glycan substitution that is critical for establishing infection both in human male volunteers and in the female BALB/c mouse model.

While the role of HepI glycans in pathogenesis has not been defined previously in the context of the murine vaginal colonization model, most human studies have used WT strains that predominantly express the 4-Hex HepI glycan structure. To our knowledge, only two studies have examined the role of pv of HepI glycans *in vivo*. In these studies, human male volunteers were experimentally challenged with a wild-type *Ng* variant whose HepI glycan was predominantly 2-Hex, with traces of 4-Hex (MS11 mkA). Recovered bacteria expressed 2-Hex HepI glycans transiently until the onset of dysuria. However, with the progression of infection, the observed HepI LOS phenotypes became predominantly 4-Hex and 5-Hex (also called the MS11 mkC variant) (199). In a separate study, the infectious dose of MS11 mkA was determined to be higher than MS11 mkC (125). The caveat in interpreting these experiments is that

although MS11 mkA predominantly expresses the 2-Hex structure from Hepl, it expresses a small amount of the 4-Hex structure too. Therefore, the contribution of only the 2-Hex structure in initiation of infection could not be ascertained. Nevertheless, these studies provided evidence that *Ng* expressing short Hepl glycans may not cause sustained infection. My studies in mice using LOS mutants with non-variable structures strongly suggest that longer (4- and 5-Hex) Hepl glycans are necessary for durable infection, and confirm the findings of Schneider and colleagues in human male volunteers.

The simplest model of STI dynamics states that $R_0 = \beta Dc$, where R_0 is the average number of secondary cases of infection generated by a primary case in a susceptible population, β is the probability of transmission within a sexual partnership, c is the number of new sex partners the infected person has in a unit of time and D is the duration the person is infectious (202, 203). An $R_0 > 1$ implies that an infection will persist, while an $R_0 < 1$ predicts elimination of infection. Thus, even a modest decrease in the duration and burden of *Ng* infection will profoundly impact transmission. We therefore posit that even if the 2-Hex/G+ and 3-Hex/G+ mutants cause attenuated infection in humans, they may not reach the threshold that is conducive for *Ng* transmission and spread within a sexual network.

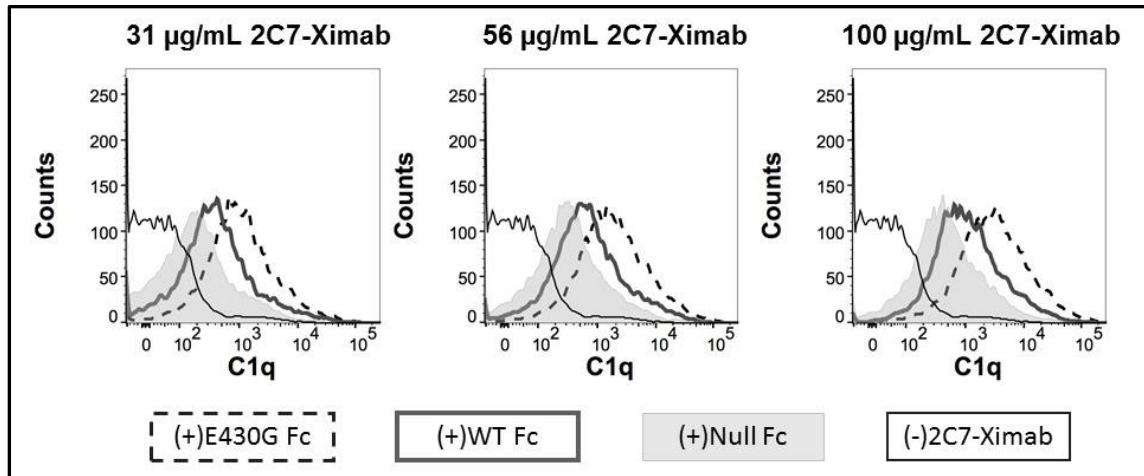
While the propensity of *Neisserial* infections in C' deficient individuals is well recognized, the *in vivo* mechanism of action of 2C7-Ximab remains unclear.

Unpublished studies demonstrate that depletion of PMNs or macrophages do not affect the ability of 2C7-murine to clear infections (Dr. Sunita Gulati, personal communication), suggesting a role for C' in clearance of infection. However, PMNs are important innate effector cells and human PMNs contribute to Ab and C'-dependent killing of *Ng* *in vitro* (22, 122, 204-210). Moreover, inherent differences between murine and human PMNs (discussed in Chapter IV) makes interpretation of the *in vivo* PMN depletion experiment ambiguous. Therefore it would be premature to dismiss the role of PMNs in clearance of *Ng* infections without further studies – for example, studies in mice that possess functional human neutrophils may help address this question (211). The requirement for the classical pathway was recently demonstrated using C1q Knock-out (KO) mice, which failed to clear infection despite 2C7-Ximab treatment. Moreover, due to the species-specific binding of C' inhibitors by *Ng* (212, 213), the efficacy of 2C7-Ximab E430G Fc was also tested in human C4BP and FH dual transgenic (Tg) mice. The dual Tg mice cleared infection albeit less well compared to WT BALB/c mice at similar doses of 2C7-Ximab E430G Fc (Dr. Sunita Gulati, personal communication).

Future studies will focus developing a fully humanized mAb 2C7 E430G Fc will likely reduce the potential immunogenicity (anti-drug Ab). Further, my studies have addressed the use of 2C7-Ximab given intra-vaginally. This is relevant for its use as an immunoprophylactic (e.g. as delivery via vaginal rings or as topical gel formulations), but not as treatment for established disease. Therefore, mouse

studies in which 2C7-Ximab is administered systemically will be carried out to investigate its use for the treatment of established disease.

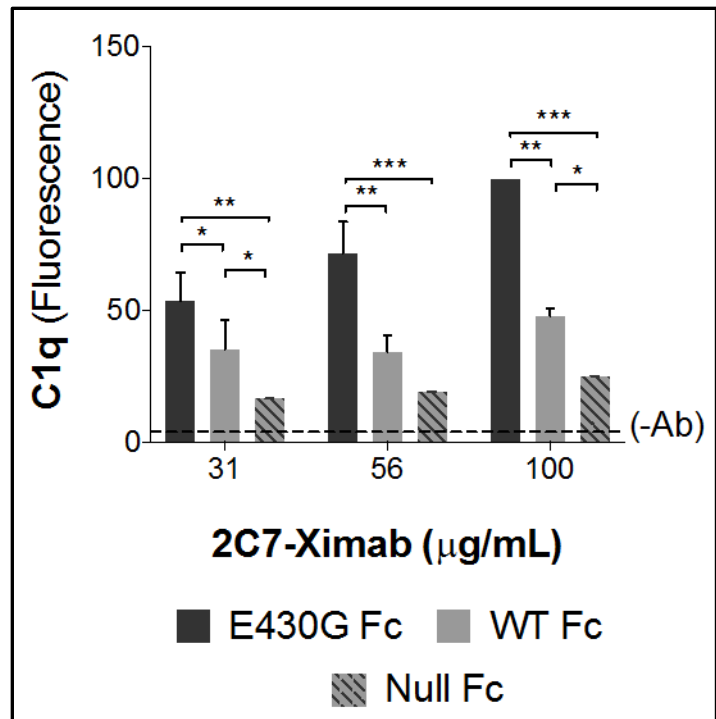
Supplementary Materials



Supplementary Figure 1. C1q recruitment by the 2C7-Ximab(s) on the 2-Hex/G+ mutant. Representative histograms of C1q deposition assays. Bacteria were harvested from overnight cultures on chocolate agar plates and sub-cultured in liquid medium for 4 h with shaking at 37 °C at 250 rpm. *Ng* were suspended to a turbidity of 0.4. Hundred µL of bacterial suspension was washed and re-suspended in HBSS^{Ca2+Mg2+} and incubated with 31, 56, or 100 µg/mL 2C7-Ximab(s) and 3 µg of purified C1q for 10 mins with shaking at 150 rpm at 36 °C. Subsequently the reaction mixture was fixed with paraformaldehyde at 37 °C with an equal volume of 2% paraformaldehyde for 10 mins. The reaction was washed with buffer and bound C1q was tagged using Biotinylated anti-C1q Ab [JL-10 clone] (Abcam). Biotinylated Ab was detected using Streptavidin-DyLight 633 (Fisher) over a BD LSRII flow cytometer.

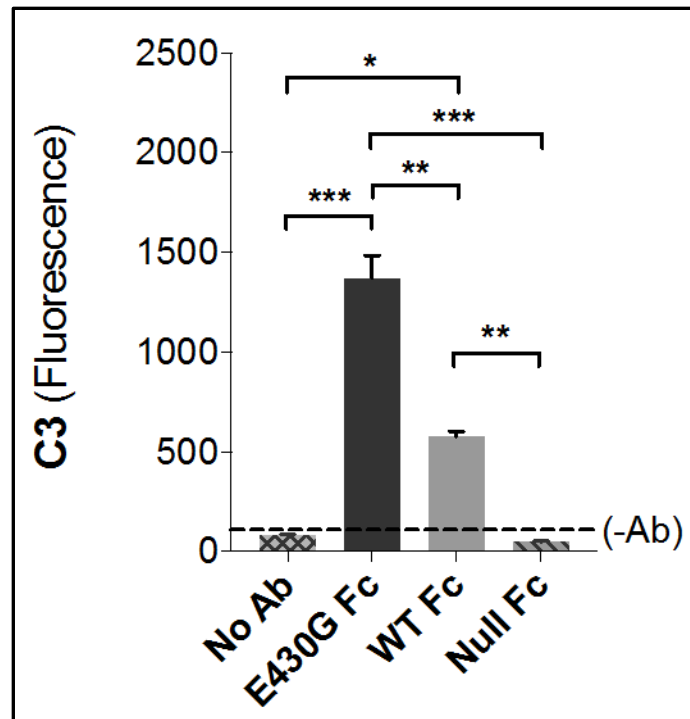
Supplementary Figure 2.

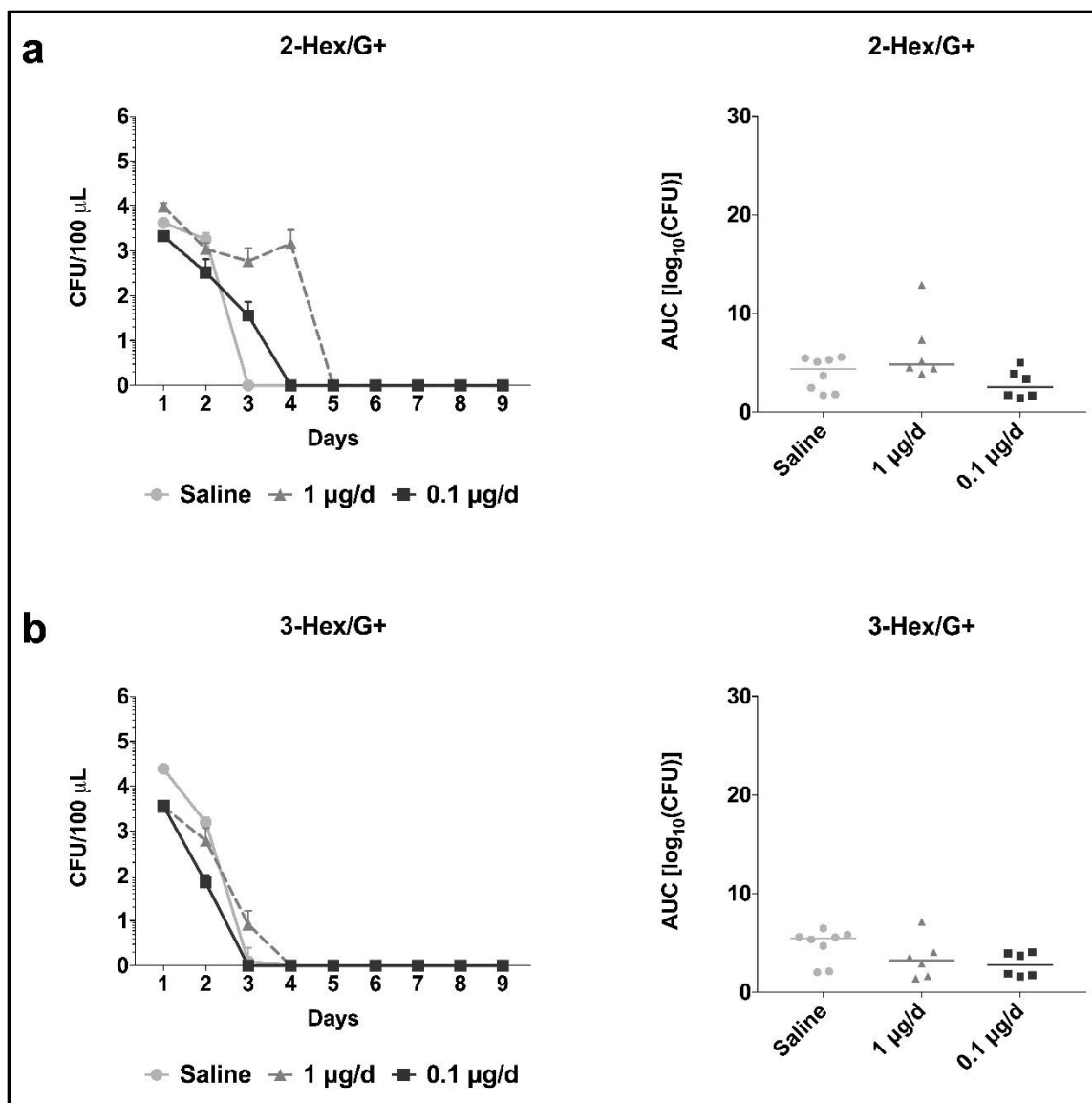
C1q recruitment by the three 2C7-Ximab(s) on MS11 ΔOpa . E430G Fc recruits significantly more C1q compared to WT Fc and Null Fc. C1q deposition assays were done as described previously. Geometric means of fluorescence were recorded and normalized to C1q deposited on bacteria opsonized with 100 $\mu\text{g/mL}$ of E430G Fc. Each bar



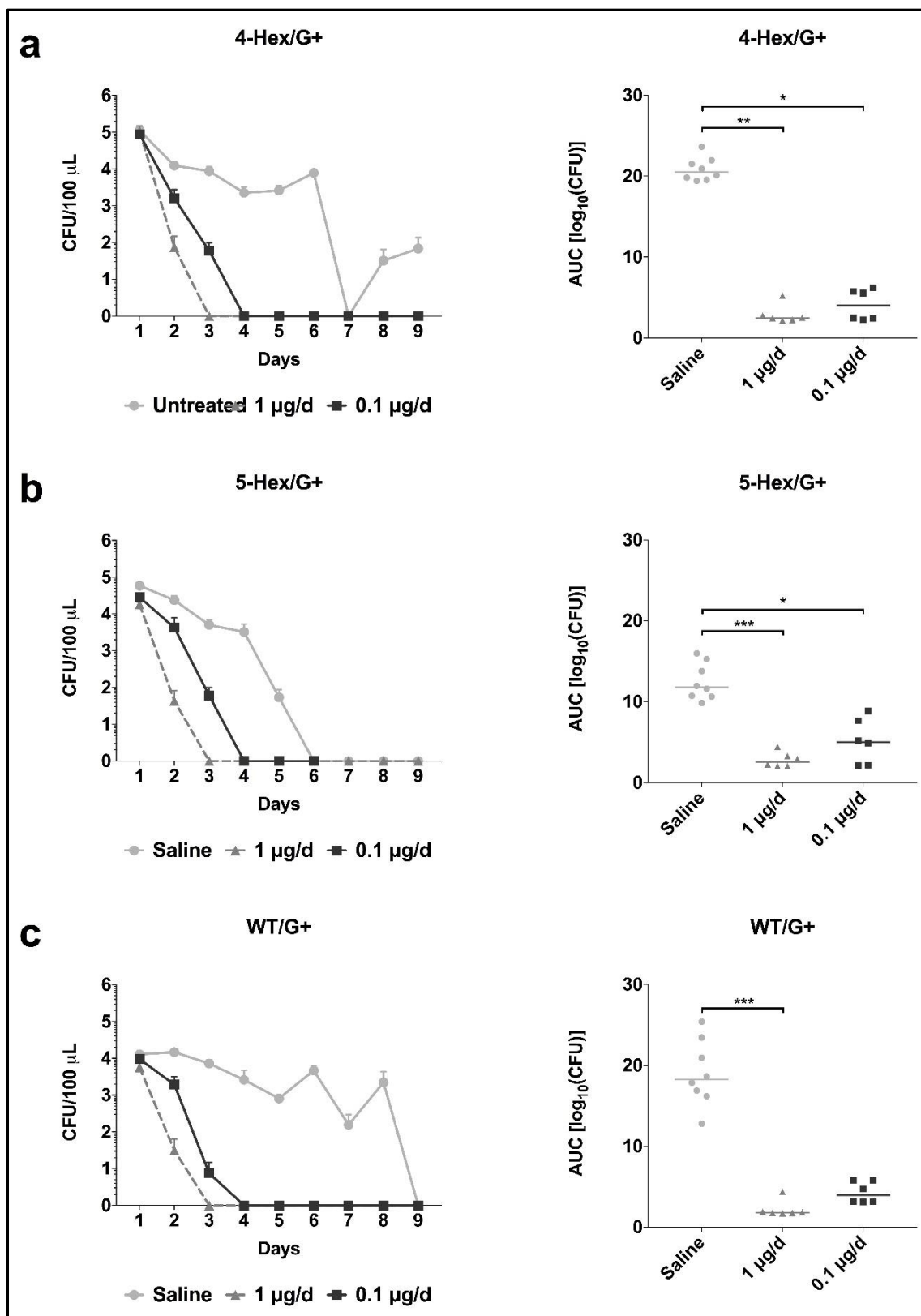
represents the mean of 2 experiments (\pm range). Groups were compared using 2-way ANOVA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). MS11 ΔOpa predominantly expresses a 4-Hex/G+ LOS (data not shown).

Supplementary Figure 3. C3 deposition by the three 2C7-Ximab(s) on MS11 ΔOpa . E430G Fc recruits significantly more C3 compared to WT Fc and Null Fc. C3 deposition assays were done as described previously. Geometric means of fluorescence were recorded. Each bar represents the mean of 2 experiments (\pm range). Groups were compared using ordinary one-way ANOVA (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). MS11 ΔOpa predominantly expresses a 4-Hex/G+ LOS (data not shown).





Supplementary Figure 4. Bacterial burdens are similar in treated and untreated groups of mice infected with 2-Hex/G+ (**a**) and 3-Hex/G+ (**b**) mutants. In the CFU/100 μ L (left graphs) each data point is the mean of all animals (\pm SEM). In the AUC (right graphs) the dash indicates the geometric mean.



Supplementary Figure 5. E430G Fc significantly reduces the bacterial burden in mice infected with 4-Hex/G+ **(a)**, 5-Hex/G+ **(b)**, and WT/G+ **(c)** mutants. Mice were treated with 2C7-Ximab E430G at doses of 1.0 µg/d, 0.1 µg/d or with saline (vehicle control). The graphs on the left show mean log₁₀ CFU vs time, while the graphs on the right show AUC (log₁₀ CFU). The horizontal lines depict the geometric means for each group. AUCs were compared using one-way ANOVA (Kruskal-Wallis) test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

CHAPTER IV

Discussion

Rationale, objectives, and salient findings of this dissertation

Globally, there are 78.3 million new cases of gonorrhea reported each year (214). Mucosal inflammation associated with gonorrhea enhances the risk of transmission, as well as acquisition of HIV (215). Moreover, gonorrhea places a significant economic burden. In 2013, the estimated direct medical costs for treating gonorrhea in the US were 162.1 million (range: 81.1 – 243.2 million) USD (216). Along with its sequelae, the cost of treatment of gonorrhea is thought to be upwards of 10 billion dollars in the United States alone. Furthermore, between 2010 – 2014, a >3-fold rise urgent care center visits were recorded for patients seeking treatment for gonorrhea in comparison to previous years (217). To make matters worse, *Ng*, the etiologic agent of gonorrhea has acquired resistance to every antibiotic approved for use against it (64). There are no safe and effective vaccines against gonorrhea available yet. Some experts predict that epidemics of drug resistant gonorrhea will become commonplace by 2020 (39). Thus, gonorrhea is a major public health concern, and there is an urgent need for the development of vaccines and novel therapeutics against *Ng*.

Vaccine development against gonorrhea has been limited by lack of interest from the pharmaceutical sector, paucity of immunodominant epitopes that elicit protective Ab(s), variable expression of Ag(s) and widespread Ag variation (66). Studies of gonococcal LOS led to the identification of mAb 2C7, a murine monoclonal that reacts with >94% of *Ng* strains (87). LOS is a ubiquitous Ag and

an important virulence determinant. Moreover, the epitope recognized by mAb 2C7 has been demonstrated to be critical for pathogenesis *in vivo* (70, 89). Active immunization with a peptide mimic of the mAb 2C7 epitope (mimitope), as well as passive immunization with the antibody itself resulted in shortened duration of infection as well as lower bacterial burdens (70). Therefore, mAb 2C7 and its mimitope are being developed as an immunotherapeutic/prophylactic and a vaccine candidate respectively.

Gonococcal LOS is a highly variable structure on account of SSM of four *pv* genes (*lgtA*, *lgtC*, *lgtC*, and *lgtG*) (122). More than 94% of the clinical strains of *Ng* express the mAb 2C7-epitope, implying that the *lgtG* is turned 'ON' (87). The Hepl phenotypes determined by the three other *lgt* genes appears to be more variable (122). Thus, mAb 2C7-reactive strains can express one or more of four distinct Hepl LOS forms: 2-Hex/G+, 3-Hex/G+, 4-Hex/G+, 5-Hex/G+. However, efficacy studies with 2C7-murine have been conducted with a relatively avirulent WT strain (FA1090) predominantly expressing one LOS structure (4-Hex/G+) (70, 125, 218).

The significance of gonococcal Hepl LOS structures other than the 4-Hex structure in virulence remains poorly understood. Thus, one of the goals of this dissertation was to investigate the effects of Hepl LOS structures on *Ng* infectivity *in vivo*. The three *pv lgt* genes controlling Hepl LOS structures of *Ng* have been evolutionarily conserved in this ancient pathogen unique to humans.

Therefore, it is reasonable to hypothesize that each structure plays a role in infection. Hence, it was important to determine whether a mAb 2C7-based vaccine or immunotherapeutic would be effective against the different HepI LOS structural variants, and whether Ab(s) targeting the mAb 2C7 epitope would be functional against more virulent isolates such as MS11. Furthermore, since recurrent *Ng* infections have been associated with C' deficiencies, we sought to answer if enhancing the C' activating abilities of 2C7-Ximab confers any therapeutic benefit.

To answer the questions in the preceding paragraph a well-studied isolate of *Ng*, MS11 was chosen as the background strain. With an infectious dose as low as 250 bacteria in human male volunteers, MS11 is more virulent than FA1090 (125). I constructed mutants in MS11 such that each mutant expressed one of the eight distinct LOS structures (other structures that result from LOS being exported to the outer membrane before all the Lgt(s) have added their substrate were also present). While four of these mutants (5-Hex/G-, 4-Hex/G-, 3-Hex/G-, and 2-Hex/G-) lacked the mAb 2C7 epitope and were used as controls, the G+ mutants that bound the Ab were used for the studies described in this dissertation because *lgtG*- (or variants that do not express HepII glycans) mutants are attenuated in mice and rarely found in nature (70, 89).

HepI LOS glycans regulated the binding of 2C7-murine. Highest binding was observed to the 2-Hex/G+ mutant, followed by the 4-Hex/G+ and the 5-Hex/G+

mutants. The 3-Hex/G+ mutant bound significantly lower amounts of 2C7-murine. Differences in Ab binding translated to differences in activation of the classical C' pathway by 2C7-murine. The 2-Hex/G+ mutant was killed by 4 µg/mL of 2C7-murine, 4-Hex/G+ and 5-Hex/G+ were killed by 10 µg/mL, while the 3-Hex/G+ remained resistant even at 10 µg/mL. Resistance of the 3-Hex/G+ was in part mediated by the binding of the classical pathway inhibitor C4BP. Blocking C4BP binding, increasing serum concentrations, or using a non-C4BP binding strain partially alleviated the serum resistance of the 3-Hex/G+ in the presence of 2C7-murine. Despite low binding of 2C7-murine to the 3-Hex/G+ mutant, there was enough C3 deposited to mediate killing by PMNs via a process that was dependent upon both 2C7-murine and C'.

Binding patterns of 2C7-Ximab(s) simulated 2C7-murine, that is 2-Hex/G+ >> 4-Hex/G+ > 5-Hex/G+ >>> 3-Hex/G+. However, the concentrations (10 µg/mL and beyond) at which 2C7-Ximab binding could be appreciated to *Ng* Hepl LOS mutants were higher than those for 2C7-murine (2 µg/mL and beyond). The possible reasons for this will be discussed later in this chapter. Of the three 2C7-Ximab(s) (E430G Fc, WT Fc, and Null Fc), the E430G Fc was effective at a log₁₀-fold lesser concentration (31 µg/mL) compared to the WT Fc (316 µg/mL). The Null Fc had no bactericidal activity. The enhanced activity of E430G Fc was a direct consequence of greater C1q (and C3) recruitment compared to the WT Fc. 2C7-Ximab E430G Fc kills three of the four Hepl LOS mutants, 2-Hex/G+ (31 µg/mL), 4-Hex/G+ (100 µg/mL), 5-Hex/G+ (178 µg/mL). The 3-Hex/G+ mutant

remained resistant even in the presence of 500 µg/mL of 2C7-Ximab E430G Fc (data not shown). The E430G Fc deposits more C3 compared to the WT Fc on the 3-Hex/G+ mutant at both 100 and 200 µg/mL. Nonetheless, even at 100 µg/mL, the E430G Fc deposits enough C3 on the 3-Hex/G+ mutant to promote PMN mediated killing, whereas the WT Fc does not. Thus, even though statistically not significant, the higher level of C3 deposited by the E430G Fc (compared to WT Fc) may be functionally relevant.

In vivo, the duration and burden of infection caused by the WT/G+, 5-Hex/G+ and 4-Hex/G+ were shortened by intravaginal administration of 2C7-Ximab E430G. In contrast, the 2-Hex/G+ or 3-Hex/G+ mutants were significantly attenuated mice; administration of 2C7-Ximab E430G Fc did not further attenuate infection. This is probably because the 4-Hex HepI LOS structure present in all three of the more infective mutants (4-Hex/G+, 5-Hex/G+ and WT/G+) get sialylated which may confer a survival advantage in the murine colonization model (180). It is worth noting that inoculation of human male volunteers with (pre)sialylated MS11 reduced infectivity (219); excessive sialylation of the LOS of the inoculum may impair ability of the bacteria to bind to ASGP-R on male urethral cells.

Significance of findings: novel insights into mAb 2C7 biology

The lengths of *Ng* HepI LOS glycans affect murine and chimeric mAb 2C7 binding. Two lactoses, one β -linked from HepI and another α -linked from HepII together comprise the mAb 2C7 epitope. It is plausible to hypothesize that the space created by the combination of these α - and β -linked lactoses create a pocket into which mAb 2C7 can enter and bind. As the length of the HepI chain with only β -linked Hex extensions (2-, 4- and 5-Hex) increases the Ab binding declines. This is probably due to steric hindrance which occludes the mAb 2C7 binding pocket preventing entry and binding of the Ab. The terminal Gal in the 3-Hex/G⁺ mutant which showed minimal Ab binding, is α -linked. α -bonds (axial bonds) are more rigid and offer lesser degrees of freedom of movement compared to β -linkages (equatorial bonds) (220). Therefore, the terminal Glc in 3-Hex/G⁺ that extends perpendicularly from the plane of the 2-Hex HepI structure probably physically obstructs the mAb 2C7 epitope. The reader should note that the terminal α -linked HepI Hex extension in the 3-Hex/G⁺ mutant is in a different plane compared to the α -linked lactose on HepII. Thus, the two α -linked moieties may contribute differentially to antibody binding; the latter facilitating the creation of a binding pocket and the former obfuscating the pocket. A mAb, B5 specific for the inner core of *N. meningitidis* recognizes strains with short (only a Glc, or a lactose) LOS Hex extensions from HepI and the phosphoethanolamine (PEA) at position 3 (endocyclic) of HepII. However, B5 reactivity is lost in strains with longer HepI extensions or with PEA at position 6 (exocyclic) probably

because of steric hindrance (221-223). Steric hindrance is also thought to inhibit Ab binding to core LPS structures in members of the Enterobacteriaceae family when rough (core OS only) LPS molecules are substituted with smooth (with repeating O-polysaccharides) LPS (224). Similarly, *Coxiella burnetii*, the causative agent of Q fever, expresses two forms of LPS, Phase I (smooth) and Phase II (rough). It has been demonstrated that Phase I LPS sterically hinders the binding of specific Ab(s) to Phase II Ag(s) (225).

Low binding of a mAb 2C7-based vaccine or therapeutic Ab to some HepI glycans such as the 3-Hex/G+ could potentially pose an obstacle to mAb 2C7 efficacy. Escape variants of multi-drug resistant *Ng* could precipitate outbreaks of gonorrhea that are untreatable/difficult to treat. The Pneumococcal Conjugate Vaccine 7 (PCV7), which targets the capsular polysaccharides of *Streptococcus pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F was introduced in the US in 2000. However, shortly thereafter, the incidence of invasive pneumococcal disease in children below five years caused by non-vaccine serotypes, such as 19A increased (226). Similarly, vaccination or infection with Phase I *Coxiella burnetii* elicit Ab(s) reactive with both virulent Phase I organisms (late Ab[s]; >20 days) as well as avirulent Phase II organisms. However, vaccination with Phase II organisms induced Ab(s) that reacted only with Phase II organisms (225).

Lack of bactericidal activity of mAb 2C7 against the 3-Hex/G+ mutant led to the description of an alternative mechanism of action for this Ab. Neutrophils infiltrate

the genital mucosae in large numbers in symptomatic gonococcal infections. *Ng* can survive and replicate within PMNs (22, 204-206, 208, 209). However, complement C3 mediated targeting of *Ng* to PMNs results in their killing (122). Sufficient C3 is deposited on 3-Hex/G+ by mAb 2C7 to cause killing by freshly isolated human PMNs. The importance of C3 opsonization in killing of the 3-Hex/G+ by PMNs was further underscored in experiments that compared C3 deposition and PMN killing between two 2C7-Ximab(s). Statistically insignificant enhancements in C3 deposition by the E430G Fc (compared to WT Fc) resulted in functionally significant differences in C3 dependent killing of the 3-Hex/G+ mutant by PMNs. Previous studies have demonstrated that mAb 2C7 opsonization results in phagocytosis (without demonstrating killing) of *Ng* (227). However, studies described here demonstrate that Ab opsonization alone is not sufficient for killing. Activation of complement by mAb 2C7 is necessary for killing of *Ng* by PMNs. This knowledge can be harnessed to optimize the formulation of the candidate vaccine for maximum possible C' activation to circumvent potential escape mutants (3-Hex/G+; discussed later in this chapter).

The poor binding 2C7-murine to the 3-Hex/G+ mutant led to the animal studies to define the infectivity of the four individual HepI LOS mutants. While data gathered from the prior experimental human infection study demonstrates the propensity of *Ng* to switch to longer LOS glycans (199), experiments described in this dissertation demonstrate that even if a population of 3-Hex/G+ LOS expressing *Ng* arose under selection pressure, such variants may not be able to

establish a sustained infection. Furthermore, durable infections by longer HepI LOS glycan expressing mutants as well as higher bacterial burdens demonstrate that shorter HepI LOS glycans confer no advantage at least in the initial stages of infection. Moreover, infection in untreated animals with the 2-Hex/G+ and 3-Hex/G+ lasted for the same duration as treated animals infected with the 4-Hex/G+, 5-Hex/G+, and the WT/G+. This leads to the conclusion that there is a minimum duration before which 2C7-Ximab is effective. While the reason for this is unknown (a possible explanation discussed later in the chapter), it provides a rationale for hypothesizing that the lack of efficacy of 2C7-Ximab against the 2-Hex/G+ and 3-Hex/G+ mutants may not be a major hurdle for its clinical success. Additionally, the 3-Hex/G+ mutant can be killed by PMNs (requires both Ab and C'). The issues of pv of the LOS and its impact on a 2C7-epitope based vaccine or therapeutic had not been considered before, but the results described here justify their further development.

Limitations of this study

The genes encoding four of the LOS glycosyltransferases (Lgt[s]) are *pv*. While the genes themselves have been fixed (i.e. locked 'ON' or 'OFF') in the mutants used for the study, the levels of expression and/or activity of the four Lgt(s) could not be controlled. Thus, the 5-Hex and 3-Hex mutants also expressed shorter HepI LOS structures (hence >1 band on silver stain – Chapter II). This is because incomplete (shorter) LOS molecules are 'flipped out' to the surface before the terminal GalNAc/Gal has been added. However, such a situation will likely mimic conditions in human infection; a strain with a predominantly 3-Hex HepI LOS will express some amount of 2-Hex HepI LOS, and a strain with a 5-Hex HepI LOS likely will also express 4-Hex HepI LOS.

The 3-Hex/G+ mutant binds very little murine and chimeric mAb 2C7 compared to the other three mutants. Due to the presence of 2-Hex/G+ structures in the 3-Hex/G+ mutant, it is possible that the small amount of Ab binding observed to this mutant is a result of the presence of the 2-Hex/G+ structure that forms the 'minimal' 2C7 epitope and permits maximum mAb 2C7 binding. Similarly, the 5-Hex/G+ mutant which also expresses 4-Hex HepI LOS structures binds a little less murine and chimeric mAb 2C7 compared to the 4-Hex/G+. It is also cleared significantly faster in the mouse model compared to the 4-Hex/G+ mutant. While it may be concluded that these effects are the result of the 5-Hex/G+ LOS structure, further delineation of the effects of the 5-Hex/G+ LOS was not

possible. In humans, the terminal GalNAc of the 5-Hex HepI is a target for natural bactericidal IgM (228); whether a similar mechanism is responsible for reduced fitness relative to the 4-Hex/G+ mutant in the mouse model is not clear.

Neutrophils kill HepI LOS mutants (including 3-Hex/G+) opsonized with murine mAb 2C7/2C7-Ximab E430G Fc in the presence of C'. The exact mechanism of killing has not been explored here. Neutrophils can phagocytose complement opsonized bacteria, and subsequently phagosomes fuse with lysosomes where pathogens are killed. They can also be killed by PMNs by Neutrophil Extracellular Traps (NET). It has been shown that *Ng* can be killed by PMNs by both mechanisms (204, 229). Engagement of CR1 but not CR3 by C' opsonized *Staphylococcus aureus* and *Aggregatibacter actinomycetemcomitans* (previously *Actinobacillus actinomycetemcomitans*) have been demonstrated to upregulate NET formation by PMNs. Opsonization with IgG alone does not cause NET formation (230). It would be of interest to explore how mAb 2C7 and C' mediate killing of *Ng* by PMNs.

Further, not all three 2C7-Ximab(s) were tested in the mouse model for efficacy of clearance. Nor was the superiority of 2C7-Ximab E430G Fc demonstrated *in vivo* for this strain. These issues however, were addressed in unpublished work by Gulati S *et al.* Prior to this experiment it was demonstrated that all three 2C7-Ximab(s), E430G Fc and WT Fc cleared infections at high doses (10 µg/day given intravaginally); at this dose, the Null Fc mutant showed minimal activity; the

median time to clearance was 1 day earlier compared to saline-treated controls. At lower doses of 1 mg/day E430G Fc and WT Fc both cleared infection with similar efficacies, whereas the Null Fc showed no activity at all. Upon reducing the dose further to 0.1 mg/day, only E430G Fc, but not WT Fc, cleared infection. Therefore, only this 2C7-Ximab (E430G Fc) was chosen for studies described in this dissertation. Moreover, it has also been shown that E430G Fc fails to clear infection in C1q knockout mice (unpublished data – Gulati S. *et al.*) thereby underscoring the importance of C' in mediating clearance of *Ng*.

While intra-vaginal administration of 2C7-Ximab significantly shortened the duration of infection, the infected mice continued to shed *Ng* for 2 – 3 days. This is an observation consistent with prior active and passive immunization studies conducted with 2C7-murine (70). Why active or passive immunization does not prevent infection was outside the scope of the current study. A possible reason could be the administration of Premarin (estrogen) that was required for infection of mice. Administration of estrogen has been demonstrated not only to abrogate LPS induced production of IL-6 and IL-8, but also reverse the IL-1 β induced stimulatory effect on NF- κ B, IL-8 and TNF- α synthesis (231). TNF- α induces synthesis of C3 which is required for both the classical and alternative pathways of C' (232), whereas the synthesis of C4 (required in the classical C' pathway) is thought to be transcriptionally regulated in mice by NF- κ B (233). As discussed in the preceding paragraph, C' activation is necessary for clearance of infection by 2C7-Ximab. In light of this, it is reasonable to speculate that the suppressive

effects of estrogen (last dose administered on day +2) on the synthesis of C' proteins such as C3 and C4 delay the activation of the classical pathway by 2C7-Ximab, thereby making the mice susceptible to *Ng* infections transiently.

About 6% of circulating *Ng* strains lack the 2C7-epitope (87); loss of this epitope comes at a fitness cost (70, 89). In mice, strains capable of pv have a propensity to express the 2C7-epitope (89) and thus, it can be reasoned that in competition experiments mutants expressing this epitope have a survival advantage (70). It is also reported that *Ng* isolates lacking the 2C7-epitope are negatively selected in transgenic mice expressing CEACAM 3, 5, and 6 (89). The aims of this dissertation were determined using these rationales, however, to bolster the rationale behind the development of a 2C7-epitope based vaccine/therapeutic, it might be useful to assess the infectivity of 2C7-negative Hepl mutants in the future.

Finally, it is important to bear in mind that while one can make predictions about pathogenesis of *Ng* and the efficacy of a 2C7-epitope based vaccine or immunotherapeutic, definitive conclusions can only be made after appropriate human studies. There are several similarities between the murine vaginal colonization model and human *Ng* infections that merit the use of the model. The influx of PMNs (hallmark of clinical disease), elevated levels of IL-1, IL-6, and TNF- α are all observed in female BALB/c mice (234). Further, the pH of the cervix (site of infection in human females) and the murine vaginal pH are similar,

as are hormone regulated changes in mucosal viscosity and commensal flora (235). However, significant differences do exist between mice and humans; differences in sequences (that translate to binding differences) of CR3, FH, and C4BP, absence of CEACAM (receptors for the *Ng* surface protein Opa) being the most notable ones (235).

Future directions

Although data from previous experimental human studies and the current work strongly suggests that the 2-Hex/G+ and 3-Hex/G+ mutants will perhaps be less infective in humans, the only way to establish this firmly would be to perform experimental human infections with these mutants. Such experiments would not only provide better insights into the impact of HepI LOS glycans in establishing infection, it would also provide an estimation of the risks posed by mutants that may escape a 2C7-epitope based vaccine or immunotherapeutic.

Further, this dissertation establishes the critical role of human PMNs in killing the serum resistant (by classical pathway mediated MAC formation) 3-Hex/G+ mutants. Gulati S *et al.* (unpublished data) depleted murine PMNs and found no difference in clearance of WT FA1090 (predominantly 4-Hex/G+) infections in mice. However, a critical factor to consider in the interpretation of these results are the inherent differences between human and mouse PMNs; one such critical difference being in the phosphoinositide 3-kinase (PI3K) signaling pathway (236). PI3K plays an important role in the upregulation of C' receptors such as CR3 (237). In human PMNs there exist intracellular reservoirs of CR3 and CR1, and their expression on the surface is induced in the presence of stimuli (238). Since C' is required for 2C7-murine or 2C7-Ximab mediated killing of the 3-Hex/G+ mutants, it is possible that due to the inherent differences between murine and human PMNs, their contribution to clearance of *Ng* infections have not been

appreciated in the mice. To assess the role of PMNs in clearance of *Ng* infections in the presence of 2C7-Ximab and C', *in vivo* studies have to be conducted in 'human immune system' mice. One such *in vivo* model system where functional human neutrophils develop from CD34+ cord blood stem cells in NOD-*scid-γc*^{-/-} has been described by Coughlan AM *et al.* (211).

From a vaccine perspective, this body of work (along with unpublished work by Gulati *et al*) highlights the importance of C' in anti-*Ng* defenses. This will prove critical in formulations development. The choice of adjuvants has been demonstrated to affect vaccine induced protection. For instance, Aventis Pasteur's canarypox vector (ALVAC) – HIV + gp120 conferred protection in macaques against SIV when the vaccine was used with alum as an adjuvant but not when MF59 was used (239). Furthermore, adjuvants can also affect the isotype and subclass of IgG(s) produced; immunization of mice with the tumor Ag MUC1 peptide with GM-CSF induced IgG1 and IgG2b in WT mice and IgM in MUC1-Tg mice, whereas with SB-AS2 the same immunogen induced IgG1, IgG2b and IgG3 in both WT and MUC1-Tg mice (240). Adjuvants have the potential to direct Ab isotype, subclass, and glycosylation. Thus, during development of the mAb 2C7-based candidate vaccine, multiple adjuvants should be tested. Subsequent to *in vivo* testing of vaccine candidates, serum from vaccinated animals could then be used to isolate *Ng* specific Ab(s) in order to understand Ab subclass and glycosylation patterns that correlate with maximal protection. Further, animals should be injected at multiple sites (subcutaneous,

intra-muscular, etc.) in protection experiments to determine the route of administration of the vaccine.

The hyperactive 2C7-Ximab, E430G Fc (human IgG1) is bactericidal at a log₁₀-fold higher concentration compared to murine mAb2C7 (mouse IgG3). Multiple factors could affect functional efficacy such as Ab subclass (IgG3 activates C' better than IgG1), species and subclass specific differences in C' activation (human versus murine IgG3 or IgG1), glycosylation at N297. All these aspects merit further investigation. Further, the amount of Ab bound, itself could modulate function. While binding of 2C7-murine can be detected by FCM even at concentrations below 1 µg/mL, 2C7-Ximab binding was only appreciable at concentrations of 10 µg/mL and beyond. Thus, endeavors should be made to enhance binding efficacy during complete humanization of the mAb 2C7-based immunotherapeutic. The current 2C7-Ximab constructs are direct grafts of the murine Fv region into a human IgG1 Fc region. Since a peptide mimic of the 2C7-epitope is already available; it would be informative to co-crystallize the Ab with its epitope. In protein-protein interactions, whilst the overall interfaces can be quite large, not all amino acids contribute to the same extent. Only a small subset of contact residues has a critical effect on binding (241, 242). These amino acids can be determined by phage display using combinatorial alanine scanning mutagenesis, and subsequently optimized to enhance binding affinity of the humanized Ab (243). Alanine scanning mutagenesis has been previously used to map functional paratopes on bNAb(s) 4E11 and 4E5A against Dengue virus

(DENV) serotypes 1 – 4 (244); while 4E11 is a naturally occurring bNAbs and reacts only with DENV 1 – 3, 4E5A generated by mutating 4E11 neutralizes all four DENV serotypes (245). Such an approach may successfully enhance the binding affinity of a fully humanized mAb 2C7 thus lowering the dose required for treatment.

In the clinical laboratory, Gram-stained smears of specimens from males show predominantly PMNs with few epithelial cells. Endocervical specimens show a similar picture, but is not diagnostic. There has been no formal analysis of the inflammatory cell landscape in *Ng* infected tissues. In healthy human females, the primary inflammatory cells are PMNs, followed by Natural Killer (NK) cells. Significantly fewer macrophages are observed (246). An understanding of the inflammatory cell landscape in *Ng* infected tissues will be invaluable. Such information can be utilized to further engineer the Fc region of a humanized 2C7 to recruit multiple host defenses against *Ng*. The data can also be potentially mined to fine tune the immune response to a mAb 2C7-based vaccine by using adjuvants that direct a response for optimal recruitment of host defenses.

NK cells could be of particular interest. Perforin produced by NK cells belong to the same class of cytolytic toxins as complement C9; both possess the MACPF/CDC domain which inserts into cellular membranes. Perforin released from NK cells have been demonstrated to insert into considerably tough mycolic acid cell walls in *Mycobacterium tuberculosis* (*Mtb*). It has also been shown that

granulysin has a synergistic effect on the anti-*Mtb* activity of NK cells (247).

Compared to *Mtb*, the cell wall of *Ng* is considerable weaker, and therefore one can reason that the gonococcal outer membrane would be susceptible to perforin mediated damage. Thus, it would be of interest to assay if a vaccine Ab, 2C7-Ximab E430G Fc, or a fully humanized mAb 2C7 can mediate this activity by engaging FcγR(s) on human NK cells. This could then serve as an added redundant mechanism of action of the immunotherapeutic.

Finally, antibodies may sometimes exacerbate infections instead of alleviating them. This phenomenon is known as antibody-dependent enhancement (ADE). ADE is perhaps best appreciated with a second Dengue viral infection where the subsequent virus is of a different serotype. Nonetheless, ADE has also been reported for HIV, Ebola, Ross River virus (248). *Ng* has been shown to survive within both male and female epithelial cells (249, 250). Moreover, once inside epithelial cells, *Ng* could possibly be subsequently disseminated. FcγR(s) (FcγRII and FcγRIII, but not FcγRI) have been detected in vaginal, ectocervical, and endocervical cells (251). They have also been reported on male urethral epithelial cells (252). Therefore, it is reasonable to hypothesize that a 2C7-epitope based vaccine induced Ab, or humanized mAb 2C7 opsonized *Ng* might employ FcγR(s) as a portal of entry into cells. They may subsequently establish latent/sub-clinical infections or may be disseminated. Thus, histopathology studies in treated animals in the murine vaginal colonization model to rule out this possibility are merited.

APPENDIX

The previous chapters describe experiments and results pertinent to the aims of this dissertation. However, there are some questions that had to be answered prior to addressing my primary objectives, or that are of interest to readers, and is the subject of this section.

Fitness in vitro of mAb 2C7-reactive Hepl glycan mutants

Prior to addressing the *in vivo* fitness of the Hepl LOS mutants, I had to ensure that none of the mutants had an *in vitro* growth defect. This was assessed by comparing the growth curves of the four mAb 2C7-reactive Hepl LOS mutants with MS11 WT (Fig. 1). All four Hepl glycan mutants showed similar growth curves compared to MS11 WT.

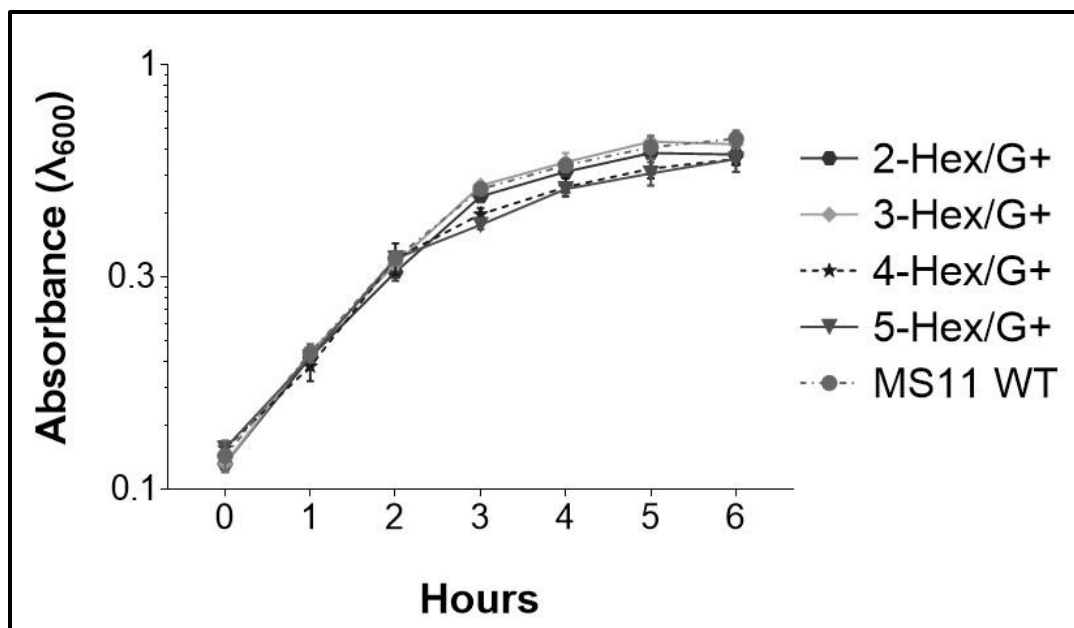


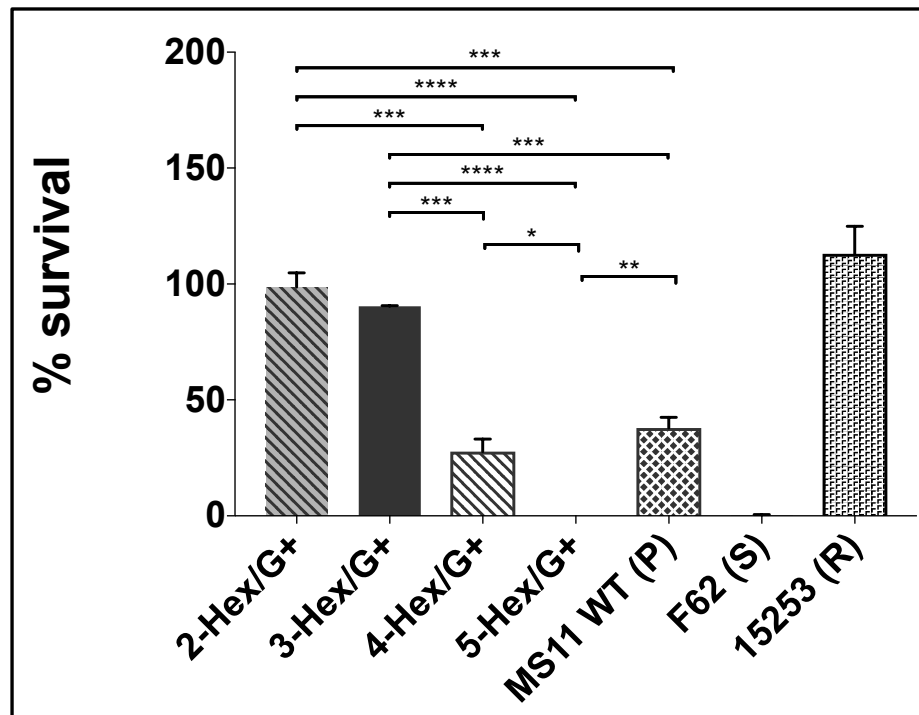
Figure 1. Growth curves of *Ng* LOS mutants. All four mAb 2C7-reactive HepI LOS mutants: 2-Hex/G+, 3-Hex/G+, 4-Hex/G+, 5-Hex/G+, as well as MS11 WT showed similar growth curves. None of the mutants had a growth defect *in vitro* in liquid medium. Overnight cultures from CA plates were suspended in complete Morse medium containing Isovitalex (9 parts Morse A, 1 part Morse B; 100 μ L Isovitalex per 10 mL complete Morse medium) to an initial absorbance (λ_{600}) of 0.1 using a Spectronic 20+ spectrophotometer. Subsequently absorbances were measured at 1 h intervals for up to 6 h. Each data point is the mean of 2 experiments (\pm range).

Serum bactericidal activity of NHS on HepI LOS mutants

Although not an objective, the ability NHS (i.e., that contains natural Ab) to activate the classical complement pathway on HepI LOS mutants was also assessed. *Ng* expressing 2-Hex/G+ and 3-Hex/G+ mutants were serum resistant whereas a mutant that expressed a terminal GalNAc (5-Hex/G+ in my studies) was completely sensitive (Fig. 2). Mutants expressing the LNnT (4-Hex/G+) LOS structure showed intermediate serum resistance (Fig. 2). The MS11 WT (MS11 4/3/1) is the parent strain from which the HepI LOS mutants were derived. My results were similar to those observed previously (82, 164, 184). However, while similar interpretations can be drawn from the earlier publications, this is, to the best of our knowledge the first systematic evaluation of serum susceptibilities (to NHS) of *Ng* expressing fixed HepI LOS structures using mutants in an isogenic background.

Figure 2.

Serum bactericidal assays with 20% NHS. In the absence of any exogenously added Ab(s), the 2-Hex/G+ and 3-Hex/G+ mutants are resistant to C'-mediated



bactericidal effects of NHS. The 4-Hex/G+ mutant was intermediately resistant and the 5-Hex/G+ mutant was completely killed. The parent MS11 WT strain was also intermediately resistant (F62 – sensitive control, 15253 – resistant control). Each bar represents mean of 2 replicates (\pm SEM) using two different NHS sources. Comparisons between mutants were made using ordinary one-way ANOVA (Tukey's multiple comparison test). Overall P value was <0.0001 . (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). Serum bactericidal assays were done as described previously. Briefly, about 2000 CFU of bacteria were incubated with 20% NHS for 30 min at 37 °C with 5% CO₂ without shaking for 30 min. Aliquots were plated in duplicates on chocolate agar at T= 0 and 30 min. Colonies were counted the following day and the number of colonies at T30 were expressed as a percent of the number of colonies at T0 to calculate survival.

HepI LOS glycans regulate binding of factor H to *Neisseria gonorrhoeae*

FH is a negative regulator of the alternative C' cascade. C3b deposited by the classical pathway can recruit the alternate complement pathway positive feedback loop. Thus, it was important to assess binding of FH to the HepI LOS mutants. HepI LOS mutants regulate binding of FH to *Ng*; FH binding decreases as HepI glycan length increases. The differences however, were significant only for the 2-Hex/G+ mutant (Fig. 3). Nonetheless, mAb 2C7, which binds best to the 2-Hex/G+ mutant, activates the classical pathway sufficiently to overcome the effects of FH. Similar trends were observed with C4BP (negative regulator of the classical cascade). I hypothesize that longer HepI glycan structures may sterically hinder binding of FH and C4BP to their ligand(s) on the surface of *Ng*.

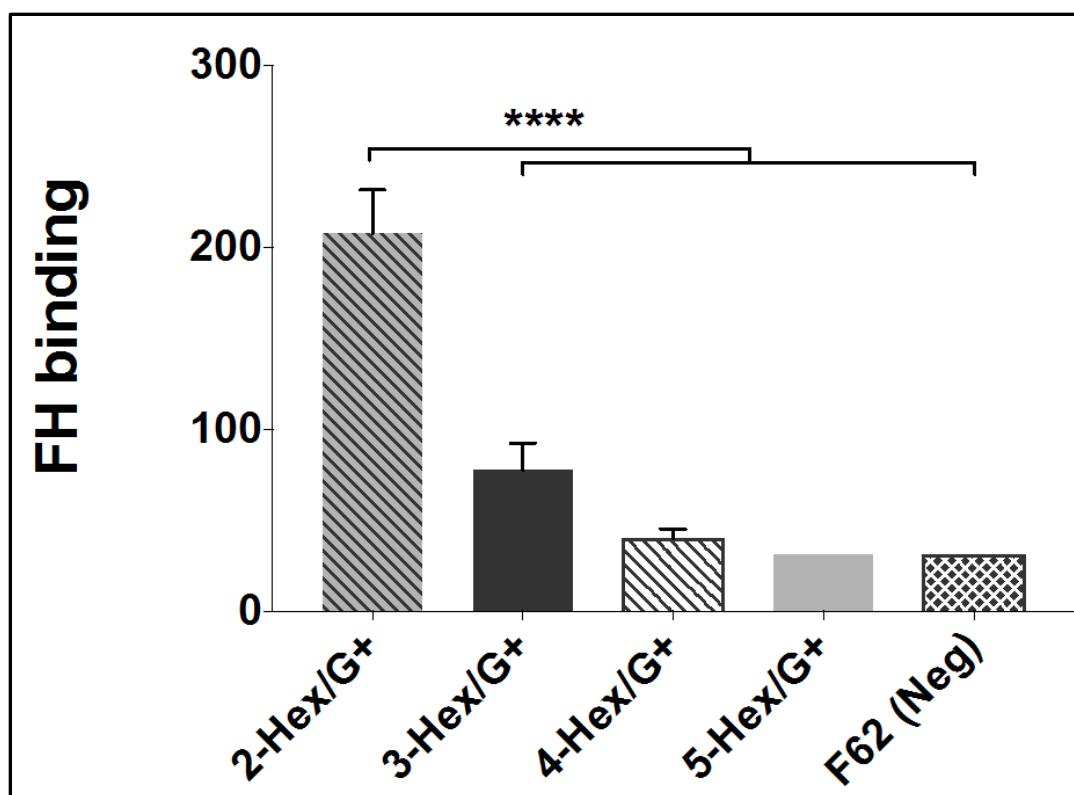


Figure 3. FH binding to the MS11 *lgtG* 'ON' (G+) mutants. HepI LOS glycans modulate FH binding. FH binding declines as HepI LOS glycan length increases. Significantly higher binding is observed to the 2-Hex/G+ mutant compared to the other HepI LOS mutants. Bacteria were incubated with 20% heat inactivated human complement for 30 min at 37 °C and bound FH was detected with polyclonal goat anti-human FH, followed by biotinylated anti-goat IgG (Molecular Probes), followed by Neuravidin conjugated DyLight 633 (ThermoFisher Scientific). Fluorescence was measured using a BD LSRII. The number alongside each bar represents the median fluorescence intensity. Each bar is the mean of 5 replicates (\pm SEM). Mutants were compared using ordinary one-way ANOVA. Overall P value <0.0001 (****, $P < 0.0001$).

Effect of sialic acid on mAb 2C7 function

The LNnT (4-Hex) LOS structure of *Ng* becomes sialylated *in vivo*. Sialylation of the LNnT enhances FH binding to *Ng* that inhibits the alternative pathway. C3b deposited by the classical pathway activates the alternate pathway. Therefore, C' activation on the 4-Hex/G+ mutant by mAb 2C7 was studied when bacteria were grown in the presence of increasing concentrations of CMP-Neu5Ac. I observed a progressive increase in serum resistance with increasing concentrations of CMP-Neu5Ac. 2C7-murine at a concentration of 4 µg/ml killed approximately 50% of the 4-Hex/G+ mutants grown in the presence of 2 µg/mL of CMP-Neu5Ac (Fig. 4). The mutant becomes fully resistant when grown in presence of 40 µg/mL of CMP-Neu5Ac (data not shown).

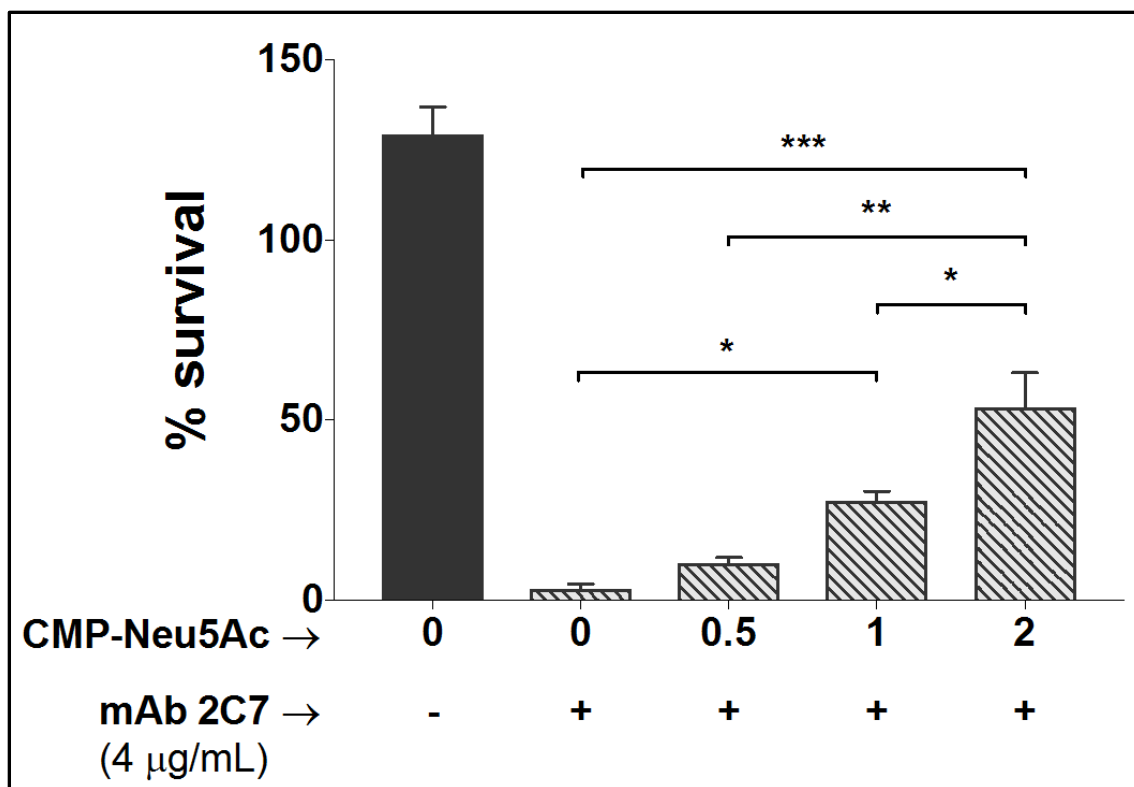


Figure 4. Serum bactericidal activity of 2C7-murine on sialylated 4-Hex/G+. Sialylation of 4-Hex/G+ makes it resistant to C' mediated killing in the presence of 2C7-murine on account of increased FH binding. Serum bactericidal assays were done as described previously with 4-Hex/G+ grown in the presence of varying amounts of CMP-Neu5Ac. Each bar is the mean of 3 replicates (\pm SEM). Groups were compared using ordinary one-way ANOVA with an overall P value of 0.0005 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Plating efficiency of Ng in the presence of antibiotics

Antibiotics sometimes decrease the plating efficiencies of bacteria. Therefore, to make comparisons across the various mutants *in vivo*, where daily CFU were determined by plating on CA supplemented with vancomycin, colistin, neomycin, trimethoprim and streptomycin (CA-VCNTS) it was important to determine the plating efficiencies of the HepI LOS mutants. There were no differences in CFU counts across the four HepI mutants or MS11 WT (Fig. 5). Further, there were no differences in the number of colonies on CA versus CA-VCNTS (Fig. 5).

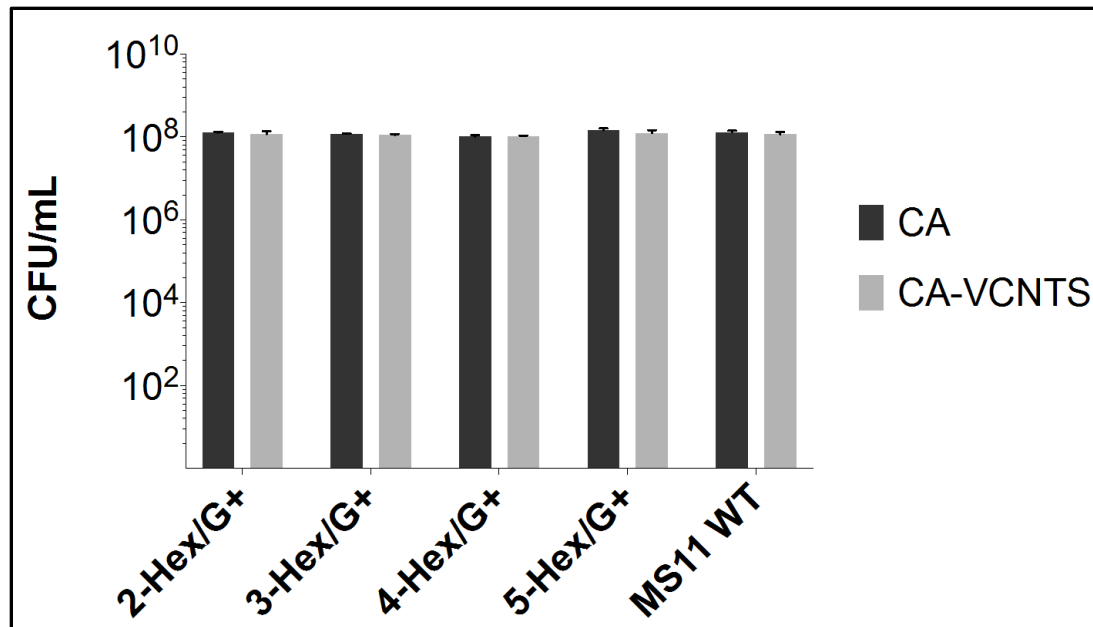


Figure 5. Plating efficiencies on CA versus CA-VCNTS agar. There were no differences in plating efficiencies when bacteria were cultured on CA-VCNTS compared to CA. The four *Ng* LOS mutants and MS11 WT were grown overnight on CA and suspended to an initial absorbance of 0.1 in normal saline (0.9% NaCl). Subsequently, the suspensions were further diluted in normal saline (1:2). Twenty μ L of this diluted suspension were plated on CA as well as CA-VCNTS agar. Twenty μ L of 10-fold serial dilutions of the diluted bacterial suspension were also plated on CA and CA-VCNTS agar plates. Plates were incubated overnight at 37 °C in the presence of 5% CO₂ and CFU were counted to determine if there were differences in plating efficiencies between the two media. Each bar is the mean of 2 experiments (\pm range).

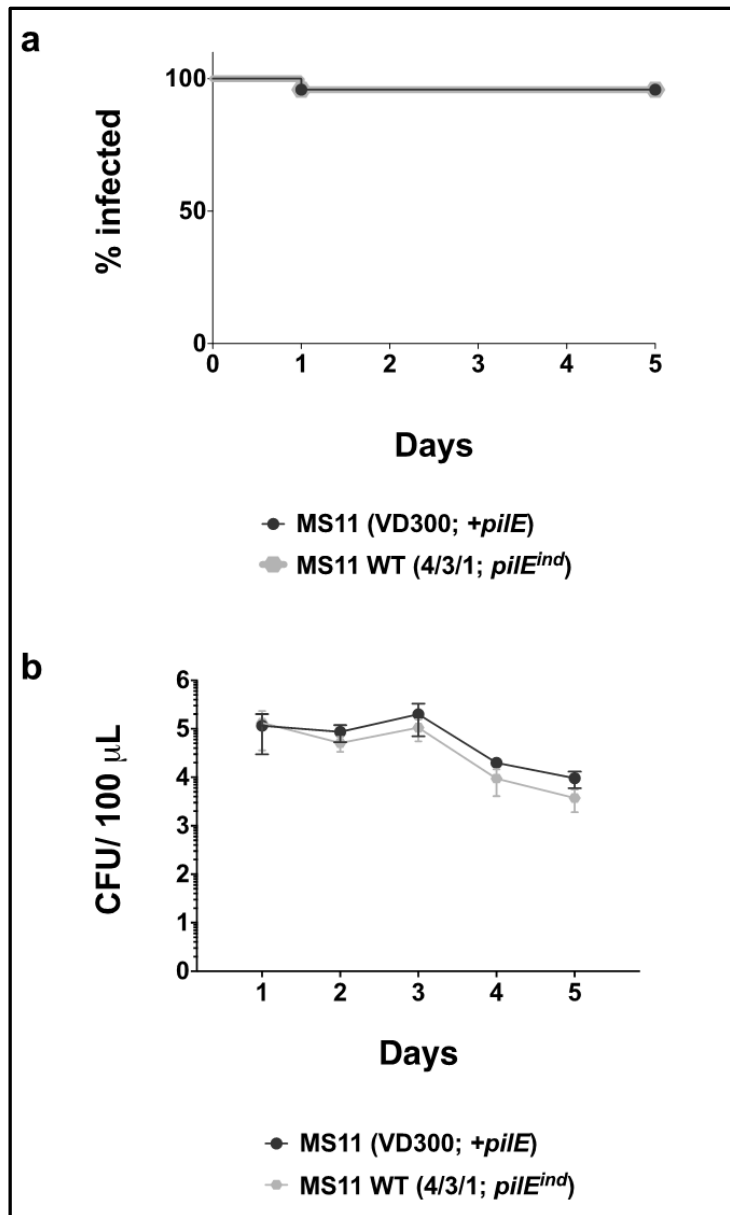
Effect of piliation on murine infection

Pili are considered to be important adhesins and are hypothesized to play an important role in human infections. Although controversial, pili are thought to bind CD46 r

ceptors expressed on the human vaginal epithelium (31). The strain of MS11 used as a background for constructing the LOS mutants in this study does not constitutively express pili, but does so when induced with IPTG (which was necessary to transform the strain). Piliation has been previously described to be inconsequential for murine vaginal infections (126). However, these experiments were conducted with non-piliated variants. Variants may start expressing pili once in the murine tract. Therefore, before the LOS mutants were constructed, a pilot mouse experiment was done to verify whether piliation is indeed unimportant in the colonization of murine vaginal tracts. The background strain MS11 WT (4/3/1; *pilE^{ind}*) remains non-piliated in the murine vaginal tract. The results confirmed previous observations. There were no differences in the duration of infection or CFU counts between pilated and non-piliated MS11 in a co-infection study (Fig. 6).

Figure 6. *In vivo* coinfection studies with pilated and non-piliated *Ng*. Pilus does not impact ability of *Ng* to colonize mice. This confirms studies conducted earlier with pilated and non-piliated variants of *Ng*. Twelve 6-8 weeks old BABL/c mice were infected as described in Chapter III with a one to one mixture of pilated MS11 (VD300; +*pilE* with an erythromycin resistance cassette) and non-piliated MS11 WT (4/3/1; *pilE*^{Ind}; background strain for mutants described in this dissertation). Vaginal swabs were plated on CA-VCNTS and CA-VCNTS-Erm (100 µg/mL). The number of colonies on CA-

VCNTS-Erm were subtracted from the number of colonies on CA-VCNTS to decipher the number of MS11 WT (4/3/1) colonies. The experiment was terminated after 5 d as: **(a)** Both the pilated and the non-piliated showed similar patterns of infection and **(b)** More or less equivalent numbers of CFU of both pilated and non-piliated mutants were recovered (Mean of CFU/100 µL [±SEM]).



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